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Ultraviolet-Induced Hemolysis of Frog Erythrocytes in the Presence of Various Nonelectrolytes[1]

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The mammalian erythrocyte has for many years provided an excellent experimental tool for the study of cell permeability (Ponder '48) and in fact is still one of the most studied cells in this regard. Recently, investigations of the permeability changes induced by ultraviolet radiation have been added to the numerous other data on mammalian erythrocyte permeability (Leu, Wilbrandt and Liechti, '42; Cook, '56; Green, '56). Potassium loss and sodium gain by the erythrocyte following ultraviolet radiation (Green, '56) as well as ultraviolet-induced hemolysis (Leu, Wilbrandt and Liechti, '42; Cook, '56) have been demonstrated.

Hemolysis of the erythrocyte from lower vertebrate forms has received less attention (Ponder, '48) even though large variations in red cell permeability have been demonstrated among lower vertebrate species (Jacobs, Glassman and Parpart, '51). The presence of an active oxidative metabolism (Hunter and Hunter, '57) and its contribution to the maintenance of the cation transport system (Maizels, '54), and the presence of a nucleus sufficiently distinguish the lower vertebrate erythrocytes from the mammalian red cell to warrant further study of these lower vertebrate cells. It would not be justified, however, to discard the wealth of information gathered for the mammalian erythrocyte as not at all applicable to the lower vertebrate erythrocytes, e.g., concepts of membrane structure (Parpart and Ballentine, '52).

In the present investigation, ultraviolet induced hemolysis of the frog erythrocyte was observed as it occurred in the presence of various alcohols.

METHODS

Blood drawn from the aorta of freshly pithed frogs (*Rana pipiens*) was heparinized, diluted about 5 times in Ringer's solution,[2] centrifuged and the supernate plasma discarded. The cells were washed two times in 10 volumes of Ringer's solution and finally suspended in Ringer's solution in a dilution of 1:100 by volume. The frog erythrocyte is relatively fragile so that centrifugation and resuspension were accomplished as gently as possible.

Methyl, ethyl, propyl, butyl, and amyl alcohols and propylene glycol were each diluted to a final concentration of 0.219 M with distilled water. This concentration was isosmotic with the Ringer's solution.

Equal volumes of each alcohol and the erythrocyte suspension were mixed so that the erythrocytes were then suspended in a solution isosmotic with Ringer's solution and consisting of 0.109 M alcohol and 0.059 M ions. The concentration of cells in this solution was 1:200. One sample of cells was diluted with Ringer's solution and was isosmotic with respect to salt ions. Another sample was mixed with an equal volume of distilled water and was 0.5 isosmotic. This 0.5 isosmotic sample was a necessary control since the alcohols quickly reach equilibrium across the cell membrane. The cell then acts as if it were in the salt solution present (Davson, '52) which in the case of the alcohols was 0.5 Ringer's solution. In a few experiments, isosmotic sucrose (0.219 M) replaced 0.4 of the Ringer's solution.

Drops from each mixture were put on quartz slides for irradiation and sealed under vaseline-edged coverslips while control drops were put on glass slides and

[1] This work was performed during the tenure of a National Science Foundation Summer Fellowship.

[2] NaCl, 0.111 M; KCl, 0.002 M; $CaCl_2$, 0.001 M; Phosphate buffer, 0.001 M; $NaHCO_3$, 0.0001 M; glucose, 0.0027 M; pH 7.4.

sealed in the same manner. Ultraviolet from a G.E. 15 watt germicidal lamp was directed at the bottom of the quartz slides from a distance of 5 cm usually for two minutes. Time from the mixing of erythrocytes and alcohols to irradiation varied between 15 and 30 minutes. The slides were kept at room temperature (24°C to 26°C) and the cells observed under low power at 30 minute intervals. For each preparation, a microscopic field was selected and the cells present in the field counted with the aid of a Whipple disc in the ocular. At each 30 minute interval, the same field was observed and the percentage of cells that had become hemolysed ascertained. Three hundred to 500 cells were included in each count.

RESULTS

The plotting of the per cent of hemolysed cells in the cell population against time following ultraviolet irradiation produces an S-shaped curve (fig. 1). Immediately following ultraviolet irradiation, no hemolysis was apparent; the cells appeared ovately discoid and exhibited the banding described by Trotter ('56). This configuration persisted for varying lengths of time from one experiment to the next at the same ultraviolet dose and was dose dependent (fig. 1). The onset of hemolysis was marked by alteration of most of the cells from discoid to a nearly spherical form. Once begun, the rate of hemolysis in the cell population increased rapidly with the inflection of the S-shaped curve at about 50% hemolysis. The abrupt onset of hemolysis and the relatively steep hemolysis curves suggests a reasonably uniform population of cells. It is possible that most of the more fragile cells hemolysed and were lost during the washing process as the centrifugal forces were kept at a minimum. It should be noted that the hemolysis rates do not follow the dose square relationship described by Cook ('56). This will be dealt with in a subsequent publication. Control samples (no ultraviolet) showed no hemolysis even after 9 to 10 hours incubation. By this time, the controls had notable bacterial

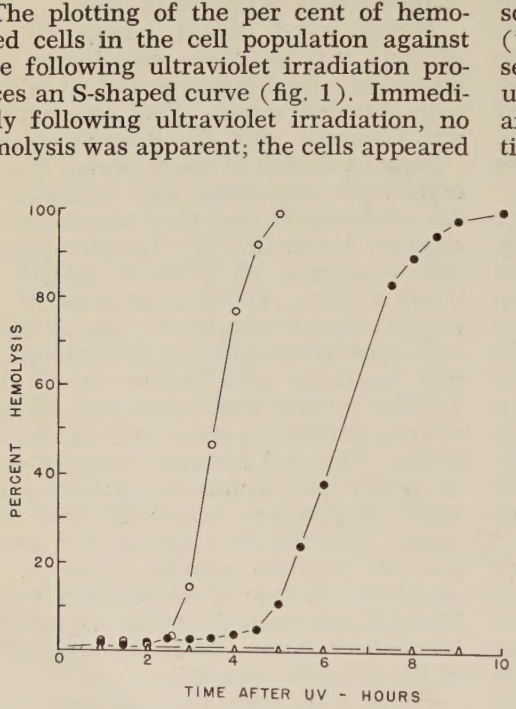

Figure 1

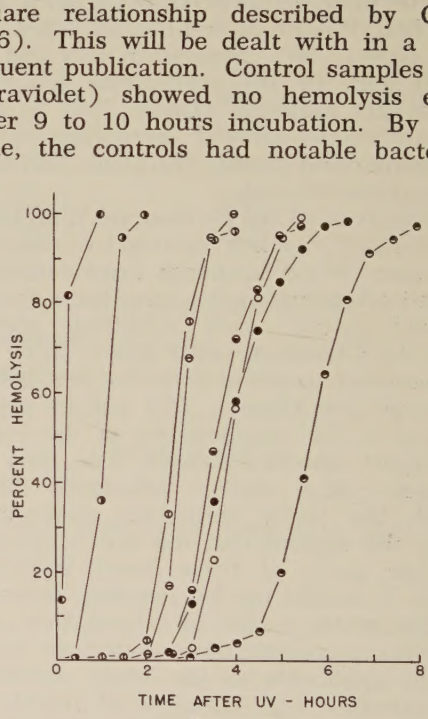

Figure 2

Fig. 1 Hemolysis following ultraviolet irradiation. ○, 4 minutes UV, Ringer's solution; ●, 2 minutes UV, Ringer's solution; △, 4 minutes UV, isosmotic sucrose (0.4) plus Ringer's solution (0.6).

Fig. 2 Hemolysis of cells in the presence of various alcohols following two minutes ultraviolet radiation. ◓, Ringer's solution; ●, 0.5 Ringer's solution; ◒, propylene glycol; ○, methyl alcohol; ⊖, ethyl alcohol; ◌, propyl alcohol; ◑, butyl alcohol; ◐, amyl alcohol; The amyl alcohol control (no radiation) hemolysed with a 50% hemolysis time of 3.8 hours (not on the graph). All other controls containing alcohol but receiving no radiation showed no hemolysis during the experiment.

growth and results beyond this time were not considered reliable. Hemolysis did not occur when isosmotic sucrose replaced 0.4 of the Ringer's solution (fig. 1).

The per cent hemolysis following ultraviolet irradiation in the presence of the various alcohols also described an S-shaped curve (fig. 2). Data for the times required for 50% hemolysis are summarized in table 1 where the alcohols are listed according to their increasing capacity for dissolving lipids. Suspending cells in a 0.5 Ringer's solution prior to ultraviolet rather than isosmotic Ringer's solution resulted in a one-third reduction of the time for 50% hemolysis. It should be noted that the alcohols which dissolve lipids less readily, i.e., propylene glycol, methyl alcohol, had 50% hemolysis times near that of 0.5 Ringer's solution. These alcohols did not increase the effectiveness of ultraviolet radiation. Ethyl, propyl, butyl, and amyl alcohols increased the effectiveness of ultraviolet in causing hemolysis according to their increasing capacity for dissolving lipids. Of these, only amyl alcohol caused hemolysis in cells not exposed to ultraviolet radiation. The slopes of the per cent hemolysis curves (between 25% and 75% hemolysis) indicated that the rate of ultraviolet-induced hemolysis was also increased when ethyl, propyl, butyl or amyl alcohols were included in the medium (table 1). The variation was large between experiments as evidenced by the large standard deviations, but the trend in each experiment was for increasing rate of ultraviolet-induced hemolysis with the increasing lipid solubility of the alcohol tested. A typical result is presented in figure 2.

DISCUSSION

Leu, Wilbrandt and Liechti ('42) and Cook ('56) offer evidence that hemolysis of mammalian erythrocytes following ultraviolet irradiation is a colloid osmotic phe-

TABLE 1

Time in hours for 50% hemolysis and hemolysis rate following two minutes ultraviolet irradiation

Treatment	Time for 50% hemolysis and standard deviation	P	Per cent hemolysis per hour and standard deviation	P
Ringer's	5.25 ± 1.25(6)[1]	< 0.05	36.2 ± 8.9(6)	< 0.01
0.5 Ringer's	3.57 ± 0.96(6)		71.8 ± 23.9(6)	
Propylene glycol	3.05 ± 0.94(4)	> 0.10	75.0 ± 33.8(4)	> 0.10
0.5 Ringer's	3.78 ± 1.17(4)		67.2 ± 25.1(4)	
Methanol	3.08 ± 0.79(6)	> 0.10	71.8 ± 29.6(6)	> 0.10
0.5 Ringer's	3.57 ± 0.96(6)		71.8 ± 23.9(6)	
Ethanol	2.46 ± 0.70(5)	< 0.05	95.0 ± 31.4(5)	> 0.10
0.5 Ringer's	3.74 ± 0.97(5)		66.2 ± 21.8(5)	
Propanol	2.07 ± 0.56(6)	< 0.01	107.7 ± 34.1(6)	> 0.05
0.5 Ringer's	3.57 ± 0.96(6)		71.8 ± 23.9(6)	
Butanol	1.10 ± 0.12(5)	< 0.01	128.3 ± 24.0(5)	< 0.01
0.5 Ringer's	3.74 ± 0.97(5)		66.2 ± 21.8(5)	
Amyl alcohol[2]	0.14 (5)	—	308 (5)	—
0.5 Ringer's	3.20 (5)		75 (5)	

[1] Number of experiments.

[2] Non irradiated cells showed hemolysis although irradiated samples always hemolysed more rapidly. Standard deviations were not determined since in two instances, the samples showed more than 50% hemolysis at the first observation following irradiation (about 5 minutes). The difference between the amyl alcohol sample and 0.5 Ringer's solution is nonetheless significant by inspection.

nomenon. For colloid osmotic pressure to cause swelling and hemolysis, the cell must be freely permeable to sodium and potassium and ultraviolet has been demonstrated to increase the permeabilty of mammalian erythrocytes to these cations (Green, '56). Ultraviolet-induced hemolysis in the frog erythrocytes also appeared to result from the colloid osmotic pressure of the cell interior. This is supported by the observation that the irradiated cell swelled in an isosmotic medium and failed to hemolyse when non-penetrating sucrose molecules were present in the ionic medium to counter the osmotic effect of the erythrocyte hemoglobin.

The more rapid hemolysis of cells in 0.5 Ringer's solution as compared to cells in isosmotic Ringer's solution may be attributed to the osmotic swelling caused by this hypotonic medium. These cells would reach the critical volume for hemolysis more rapidly because of this initial osmotic swelling. Cells irradiated in the presence of the less lipid soluble alcohols, e.g., propylene glycol, methyl alcohol, hemolysed at a rate comparable to cells in 0.5 Ringer's solution. It is concluded from this that these alcohols penetrated the cells causing the osmotic pressure within the cells to rise. Water entered the cell to compensate for the increased osmotic pressure, a process which would continue until the alcohol concentration was equal inside and outside the cell (Davson, '52). At this point the cell acted osmotically as if it were in a medium of the concentration of the ions present, i.e., 0.5 Ringer's solution, and the same explanation for more rapid hemolysis holds for these cells as for cells in 0.5 Ringer's solution (above). It should be noted that at the concentrations used, none of these alcohols caused hemolysis of the non-irradiated cells.

The hemolysis of cells irradiated in the presence of the other alcohols cannot be attributed exclusively to the phenomenon described above, i.e., osmotic swelling, although it is assumed that these alcohols also quickly reach diffusion equilibrium across the erythrocyte membrane. Ethyl, propyl, butyl, and amyl alcohols each caused 50% hemolysis following ultraviolet radiation to be reached more rapidly than 0.5 Ringer's solution, i.e., ultraviolet-induced hemolysis started sooner and the hemolysis rate was greater. Except for amyl alcohol, these effects occurred at alcohol concentrations which did not cause hemolysis of the non-irradiated cells. Parpart and Green ('51) have shown that one of the alcohols used here (butyl) can increase the permeability of mammalian erythrocytes to cations and further that swelling and presumably hemolysis depends upon the rate of cation exchange. It is postulated that in the present experiments the alcohols altered the cell membrane lipids resulting in an increased cation permeability but that this alteration was not sufficient to induce hemolysis. A constant ultraviolet damage was added to the variable alteration of membrane lipids by the alcohols to permit a cation exchange rapid enough for hemolysis at a greater rate than induced by either agent alone. The progressive enhancement of ultraviolet induced hemolysis by the alcohols according to their increased lipid solubility supports this postulate.

SUMMARY

1. Ultraviolet irradiation caused frog erythrocytes to hemolyse in an isosmotic Ringer's solution. If isosmotic sucrose replaced a portion of the Ringer's, hemolysis was prevented.

2. Dilution of the Ringer's solution by one-half shortened the time for 50% hemolysis following ultraviolet irradiation. If one-half of the Ringer's were replaced by isosmotic propylene glycol or methyl alcohol, the 50% hemolysis time was near that of the 0.5 Ringer's solution.

3. When isosmotic ethyl, propyl, butyl, or amyl alcohol replaced one-half of the Ringer's solution, ultraviolet induced hemolysis was further increased in accordance with the lipid solubility of the alcohol present. Only amyl alcohol caused hemolysis of non-irradiated erythrocytes.

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The Action of an Antimetabolite of Thiamine on Single Myelinated Nerve Fibers

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The problem of the role played by thiamine during the excitatory process in nerve has been the subject of a number of investigations (a review of the approaches to this problem and the results obtained was given by von Muralt, '58a).

With the discovery of two antimetabolites of thiamine, neopyrithiamine (Tracy and Elderfield, '41; Woolley and White, '43) and oxythiamine (Soodak and Cerecedo, '44) it became possible to distinguish two physiological roles of thiamine (Gurtner, '57). Oxythiamine is a competitor for cocarboxylase which, when administered to an animal, causes an increased content of pyruvic acid in the blood without a significant disturbance of the nervous system. Neopyrithiamine is a competitor for thiamine itself and elicits the typical symptoms observed in beri-beri, i.e., general disturbance of the nervous system. It was this evidence that led Woolley in 1954 to the conclusion that neopyrithiamine competes with some thiamine compound whose metabolic function is not mediated through cocarboxylase while oxythiamine inhibits the more general reactions in the body which require cocarboxylase. Von Muralt ('43) had postulated the existence of this second role of thiamine. From his work with Gurtner ('57) he concluded that there are two different modes of action of thiamine, one being its action in nerve metabolism, the other the enzymatic role of cocarboxylase in general metabolism.

Other antithiamine principles are known which belong to the so called structure-modifying class of antimetabolites (Somogyi, '56). Unlike the competitive replacement activity of neopyrithiamine, these substances act by modifying the structure of the thiamine molecule thus making it physiologically inactive. The antithiamine principle found in carp intestine which causes the classical Chastek paralysis (Green, '36) is an example of this type of antithiamine. This substance splits thiamine into pyrimidine and thiazol (Sealock and coworkers, '43). Another interesting example of structure modifying substances is the antithiamine principle found in fern extracts (Weswig and coworkers, '46). Both of these antithiamine substances have proved effective in experiments on nerve activity. Carp extracts and fern extracts of high antithiamine activity were found to abolish the normal response to fresh water of the water receptors in the frog when these substances were applied to the superficial endings of the glossopharyngeal nerve of the frog's tongue (von Muralt and Zotterman, '52). Konnecci and von Muralt ('49) obtained a reversible increase of threshold and a block of conduction with whole nerve bundles of frog using fern extracts.

Further advances have been made in the purification of the B_1-antimetabolite of fern by Somogyi and Kohler ('59). Two active products of this antithiamine factor, hydrolysates I and II,[1] have been obtained. In view of the availability of these two active substances, this present work on the action of fern antithiamine principle was undertaken in the hope of further clarifying the role played by thiamine in nervous activity.

MATERIALS AND METHODS

1. Preparation of single myelinated nerve fibers from the N. ischiadicus of

[1] The antithiamine substances used in these experiments were supplied by Dr. J. C. Somogyi, Director, Institut fur Ernährungsforschung, Zürich. These substances are designated hydrolysate I and II. For all practical purposes there is no physiologically significant difference in their mode of action (hydrolysate II is more active than I) and in this paper they are simply referred to as hydrolysate.

frog (*Rana esculenta*) was accomplished according to the method of Stämpfli ('52).

2. *Solutions.* The solutions used contained the following ion concentrations:

A. Ringer's solution: NaCl, 116 mM/l; $KHCO_3$, 2 mM/l; $CaCl_2$, 1.1 mM/l.

B. 20, 40 and 60 mM KCl—Ringer's. Replace 20, 40 and 60 mM NaCl with KCl, all other ions remain the same.

C. Isotonic KCl, 116 mM/l.

D. *Hydrolysate solutions.* These contained 2 mg hydrolysate per 32 ml of the various above mentioned solutions. Using the thiochrome test as a measure of the antithiamine activity of the hydrolysate solutions, Somogyi et al., ('59) showed that 1 mg hydrolysate inactivates between 19 and 21 gammas of thiamine, which is about 10 times more active than the crude fern extract.

3. Electrophysiological methods:

A. *Bridge.* The bridge used in these experiments employs the principle of the air gap between nodes N_2 and N_3 to insure a high external resistance (in the order of MΩ). If care is taken that only the myelinated portion of the internode bridges the air gap, no damage to the fiber can be observed. N_2 was isolated from N_1 and N_3 with vaseline. These two neighboring nodes were narcotized with a 0.3% cocaine-Ringer's solution. By employing the multi-valved spigot of Stämpfli (Kilb and Stämpfli, '55) the solutions surrounding N_2 could be changed with great rapidity.

B. *Stimulating and recording apparatus.* The arrangement of stimulating and recording apparatus was the same as used by Lüttgau ('54, '56a). Both the Tönnies double pulse stimulator and the Grass stimulator were employed. Potentials were recorded with a cathode follower (Tönnies No. 236–1955), a D.C. preamplifier (Cossor model 1430) and a cathode ray oscilloscope (Dumont type 304a) with a high input resistance.

C. *Action potentials and differentiated action potentials.* A stimulus of 0.44 msec. was used for the production of the action potentials and one of 2.2 msec. for the differentiated action potentials. These were applied between electrodes E_1 and E_2. The potentials were recorded between nodes N_2 and N_3. Differentiated action potentials were obtained by introducing an appropriate RC-link (200 pF and 100 k ohms) into the recording circuit. The effect of hydrolysate on the potentials was recorded after node N_2 had been bathed for 5 minutes in hydrolysate solutions.

D. *S-curves* (the measure of the rate of rise of the action potential as a function of membrane potential). This relationship, which was studied according to the method of Hodgkin and Huxley ('52), Weidmann ('55) and Lüttgau ('56b), yields an S-curve which is theoretically a measure of the inward streaming of so-

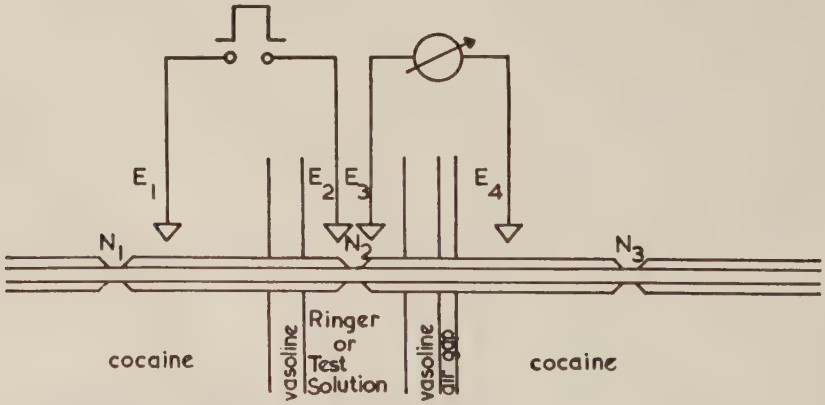

Fig. 1 Schematic representation of stimulating and recording apparatus. E_1 and E_2, stimulating electrodes. E_3 and E_4, recording electrodes. N_1, N_2 and N_3, nodes of Ranvier of single nerve fiber, isolated by vaseline. Solutions in middle trough can be changed from Ringer's solution to test solution. Nodes N_1 and N_3 narcotized with 0.3% cocaine-Ringer's solution. Air gap lies between N_2 and N_3. (The fiber with its myelin sheath is not drawn to scale.)

dium ions that can be obtained after having kept the membrane at different potential levels for a few msec. Using the Tönnies double pulse stimulator, a conditioning pulse of 20 msec. duration with varying electrical sign and magnitude was given followed immediately by a test pulse of 5 msec. Changes in the height of the differentiated action potential were recorded.

E. *Resting potential.* The resting potential was measured as the potential drop between nodes N_2 and N_3 which occurred when N_2 was depolarized with a 116 mM/l KCl solution (isotonic). The value of this potential shift was usually ⅓ to ½ of the absolute value.

F. *Membrane potential as a function of the external potassium concentration.* This relationship (Huxley and Stämpfli, '51a, '51b) was studied in the following manner: the solution surrounding N_2 was changed from Ringer's to 116 mM/l KCl to determine the amount of potential drop actually being recorded. The solution was then changed to Ringer's. When repolarization was completed, the above step was repeated with 20, 40 and then 60 mM/l KCl-Ringer's solutions. To determine the effects of hydrolysate on membrane potential and on the relationship of membrane potential to external potassium concentration, node N_2 was bathed in Ringer's and then in Ringer's plus hydrolysate for 5 min. The change in potential was recorded. Solutions were then changed from Ringer's plus hydrolysate to 20, 40 and then 60 mM/l KCl-Ringer's plus hydrolysate.

G. *Junction potentials.* Junction potentials which might arise at the recording electrode during the changing of solutions were controlled by measuring the potential between the electrode E_3 and a Ling-Gerard micro-electrode filled with 3 M KCl immersed in the middle trough of the bridge. Since no such junction potentials were observed, solutions were changed without further precautions.

RESULTS

A typical experiment depicted in figure 2 shows that bathing node N_2 in Ringer's plus hydrolysate caused an initial decrease in the action potential which slowed down and was measured after 5 minutes, starting from 59.9 mv in Ringer's to 38.3 mv in Ringer's plus hydrolysate (21.6 mv difference) and a decrease in the differentiated action potential starting from 375 v/sec. in Ringer's to 138 v/sec. in Ringer's plus hydrolysate (237 v/sec. difference). Recovery was obtained when the node had been washed out for 6 minutes with Ringer's solution.

In addition to the effect on the action potential, measurements with a DC amplifier showed a hyperpolarization of the membrane of + 7 mv (12 experiments ± 1.1 mv). The S-curves which were obtained show that the presence of the hydrolysate causes a shift to the right which is considerably larger than the effect of hyperpolarization. In addition there is a marked drop of the upper plateau of the curve such as that obtained with sodium poor solutions, c.f. figure 3a (Weidmann, '55). The zero point of the hydrolysate curve was shifted 7 mv to the right to allow for the hyperpolarization.

The addition of hydrolysate to Ringer's solution and to the solutions containing the varying concentrations of KCl resulted in a hyperpolarization over the entire range of KCl concentrations tested (c.f. fig. 4a).

Addition of an excess of thiamine (6 mg B_1 per 2 mg hydrolysate) to the solutions containing hydrolysate before applying them to the node N_2 resulted in a complete disappearance of the effects obtained with hydrolysate alone (c.f. figs. 3b, 4b). Experiments *in vitro* show that 2 mg of hydrolysate inactivate about 40 gamma of thiamine, thus the amounts of thiamine used to neutralize the antithiamine effect were quite sufficient.

DISCUSSION

According to the ionic hypothesis of electrical activity (Hodgkin, '51), the rise of the action potential results from the inflow of sodium ions into the fiber. In myelinated nerve fibers this process takes place only at the node (Stämpfli, '54). The rate of rise of the action potential (dx/dt) is a theoretical measure of this inflow (Weidmann, '55). The S-curve (dx/dt plotted against membrane potential) can be interpreted in terms of the

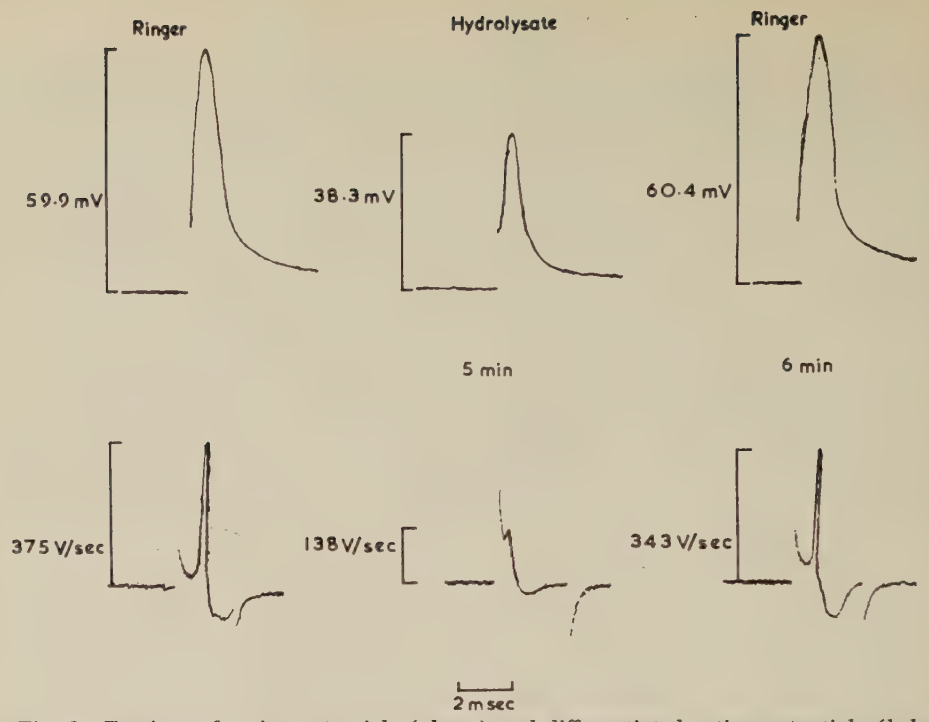

Fig. 2 Tracings of action potentials (above) and differentiated action potentials (below) showing the effect of hydrolysate (center) after 5 minutes and the recovery in Ringer's (right) after 6 minutes. Values for the action potentials are given at the left in millivolts (mv). Values for dx/dt are given in volts per second, v/sec. (Only the values for the rising phase have been used in this paper.)

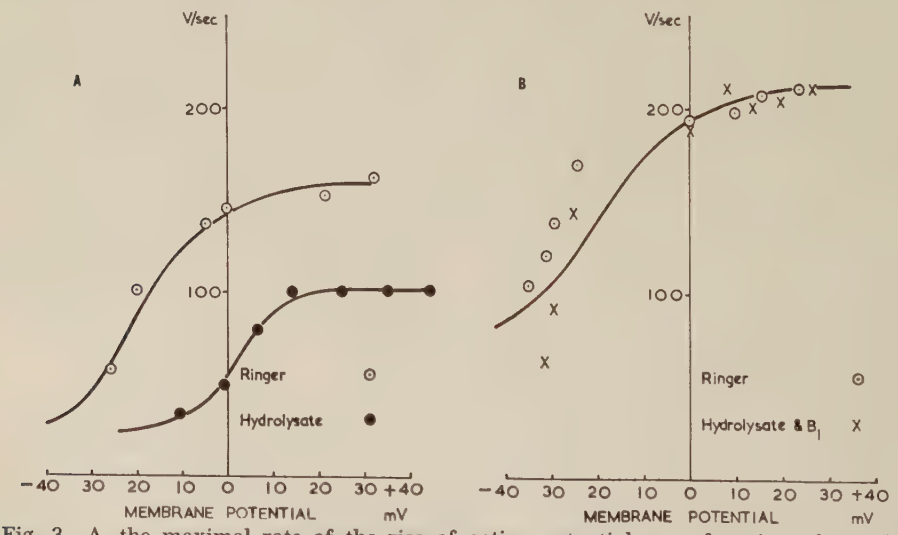

Fig. 3 A, the maximal rate of the rise of action potential as a function of membrane potential. Ordinate: maximal rate of rise (v/sec.). Abscissa: membrane potential, given as the absolute value of the change in membrane potential resulting from a conditioning impulse. Measurements were made 5 minutes after solutions were changed. A conditioning pulse of 20 msec. duration and varying electrical sign and magnitude was used to alter the membrane potential, followed immediately by a test pulse of 5 msec. S-curve with dotted circles corresponds to Ringer's solution and solid circle, to Ringer's plus hydrolysate. B, S-curve plotted in the same way as figure 3A. Circles with dot correspond to Ringer's solution, crosses to hydrolysate with an addition of thiamine.

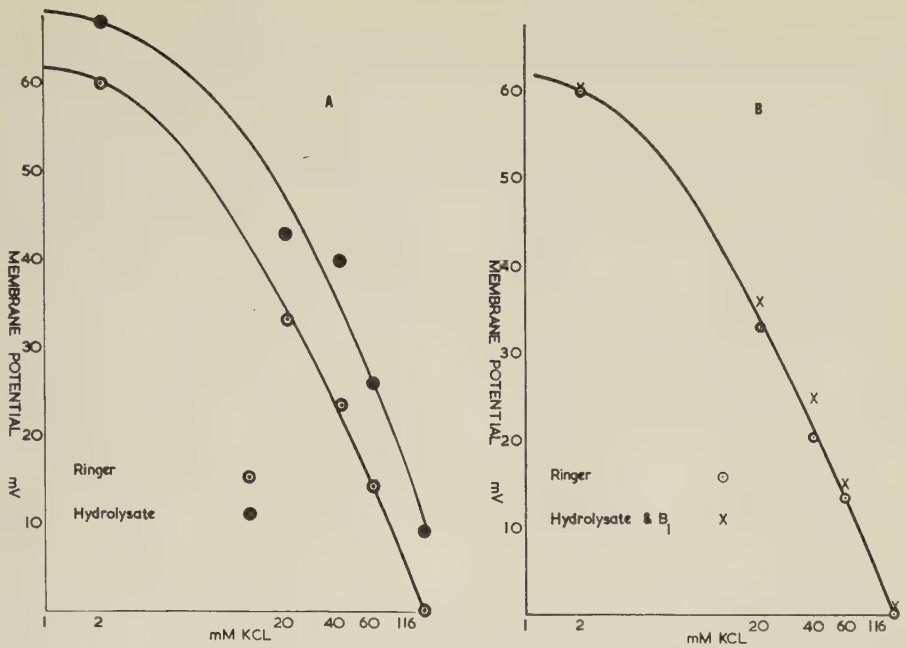

Fig. 4 A, the membrane potential (ordinate) as a function of the external potassium concentration (abscissa). Circles with dot correspond to Ringer's solutions with varying concentrations of KCl; solid circles, to hydrolysate with varying KCl concentrations. B, curve plotted in same way as figure 4A. Circles with dot correspond to Ringer's solution with varying concentrations of KCl; crosses, to hydrolysate plus thiamine with varying KCl concentrations (mean values of 6 experiments).

inactivation of the sodium carrying system (Hodgkin and Huxley, '52). The decrease in the height of the differentiated action potentials and the drop in the upper plateau of the S-curve caused by the action of the hydrolysate suggest that this antithiamine principle decreases the number of active sodium carriers available. This effect is abolished by an excess of thiamine (fig. 3b) showing that the action is mainly due to its inactivation of thiamine and not to other properties. The effect, moreover, is reversible (fig. 2) indicating that the antithiamine principle might act only by forming a loose bound complex with thiamine in the nerve which can be dissolved simply by washing with Ringer's solution.

Although this antithiamine principle of fern was at first believed to be of the thiamine destroying class of antimetabolites (von Muralt, '58), Somogyi is now under the impression that its reaction is of the thiamine complex forming type (Somogyi, personal communication). This could explain the reversibility which was found in the present experiments.

The reversibility of the reaction and the fact that it can be checked by adding an excess of thiamine to the hydrolysate are characteristics which differ from those found in the experiments with neopyrithiamine by Kunz ('56). He found that only slight reversibility of the reaction could be obtained by washing out the node with Ringer's solution and that addition of thiamine to neopyrithiamine gave no protection to the nerve. This is not in concordance with *in vivo* studies by Woolley et al. ('43) and Gurtner ('57) who found that simultaneous administration of thiamine with neopyrithiamine diminished the effects of the antimetabolite depending on the relative concentrations of thiamine to antithiamine. Thus, if neopyrithiamine acts by competitively replacing thiamine in the active sites of the organism this same type of competition is to be expected in nerve, but here there seems to be a special mechanism involved. The

protective action of thiamine in the experiments with hydrolysate is assumed to result from an inactivation of the antithiamine principle of fern.

According to the ionic hypothesis, the membrane potential is, in first approximation, the potassium potential. The magnitude of the membrane potential depends on the concentration ratio of K^+ between inside and outside and of Cl^- between outside and inside and the absolute temperature, according to the Goldman formula ('43) as applied by Hodgkin and Katz ('49). In order to explain the hyperpolarization caused by the hydrolysate according to this hypothesis, three possibilities might be considered: (a) The action of the hydrolysate may be due to a binding of potassium ions in the immediate vicinity of the excitable membrane by the antithiamine molecule thus decreasing the external K^+ concentration and yielding a hyperpolarization. This is an explanation of the hyperpolarization which does not involve the action of the hydrolysate on thiamine in the nerve. (b) The inactivation of thiamine by the antimetabolite might act by increasing the internal K^+ concentration and thereby the membrane potential. This would help to explain why, at external KCl concentrations of 116 mM/l (isotonic) where the internal K^+ concentration should equal the external K^+ concentration, there is still a hyperpolarization indicating that the internal K^+ concentration is greater than 116 mM/l. (c) The hyperpolarization may also be explained by assuming that the inactivation of thiamine causes a change in the Cl^- permeability at the resting potential but this version would not explain the shift at external isotonic KCl concentrations.

At present, no decision can be made between these possibilities and no inference is made that these are the only possible explanations.

In order to explain the effect of hydrolysate on the sodium carrying system our present knowledge is not sufficient to offer any conclusions. The experimental fact as such is interesting and may be explained in the light of further work.

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Analysis of the Spectrophotometric Reflectance Response of Frogs to Melanophore Hormone[1,2]

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Chromatophore responses have traditionally been evaluated by observation of the position of pigment granules in the cells, the position being sometimes assigned a number on an arbitrary scale described by Hogben and Slome ('31). In searching for an objective method of measuring melanosome migration within melanophores of frogs, certain objective techniques have been introduced. These were reviewed by Thing ('52), who devised a reflection photometer to measure responses in living *Rana esculenta* and compared the results with the melanophore index. Shizume, Lerner and Fitzpatrick ('54) measured responses in isolated skin of *Rana pipiens* with a reflection photometer, a method which was compared with similar measurements in intact frogs by Deutsch, Angelakos and Loew ('57). Rigler and Holzbauer ('53) have measured the relative absorbance of isolated skin.

This communication contains basic information concerning the physical aspects of amphibian color change and its relationship to melanophore hormone, as revealed by the use of spectrophotometric directional reflectance.

METHODS

Representatives of two genera of anura, *Hyla cinerea cinerea* (Schneider) and *Rana pipiens pipiens* Schreber, were studied. All animals were hypophysectomized by the method of Hogben ('22) before use. The usual pallor of the skin was noted soon after the operation and the animals were unable to darken in response to overhead illumination when placed on a black background.

Spectral directional reflectance was measured with the Beckman model B spectrophotometer equipped with a reflection assembly, a photomultiplier attachment, and a Sorensen model 1001 voltage regulator. The reflection assembly was mounted with the sample aperture upwards. Over the aperture was placed a copper platform, painted black and made to fit on the assembly. A circular hole approximately 6 mm in diameter was drilled in the platform for the incident light beam. The lid of the instrument was closed, with the frog lying supine on the platform, when a reading was taken.

The frogs were immobilized before being read by immersion in 2–5% urethane. The skin was kept moist and excess water was removed by blotting before each reading. Roughly the same area was read on each occasion; a mid-line region approximately in the center of the dorsum of *Hyla* and background areas between fixed pigmented spots of *Rana* were used. The animals were allowed to remain on the platform for only a few seconds at one time. We have previously reported that readings under urethane do not differ significantly from those of unanesthetized *Hyla;* variations in the readings of different areas of the dorsum, variations of the same area during one day, and variations from day to day have been noted (Patton and Teague, '59).

U.S.P. Posterior Pituitary Reference Standard Powder was used as the melanophore hormone preparation. It was extracted with 0.25% acetic acid and a solution was prepared, according to the U.S.P. Reference Standards Committee recommendations, to contain 2 U.S.P. Posterior Pituitary Units per ml. Injections of 0.1 ml volumes were made into the ventral lymph sac approached *via* the hind limb,

[1] Aided by grant C-3101 from the U.S.P.H.S.

[2] Preliminary abstracts have been published (Teague, R. S., and J. R. Patton: Federation Proc., *17:* 414, 1958; *18:* 450, 1959).

using a one-inch, 27 gauge needle. The solution was diluted appropriately with distilled water, and stored at 5°C.

Magnesium carbonate was used as the primary standard for reflectance measurements. Since the reflectance of the dorsum of hypophysectomized frogs is extremely low when read against magnesium carbonate, we have introduced the use of secondary reflectance standards. For the secondary standards, we have employed the Munsell series of neutral value papers, which have zero chroma and reflect no hue.[3] At a given wavelength, the product of the reading of the secondary standard against magnesium carbonate and the reading of the sample against the secondary standard gives the reflectance of the sample. In some cases, when a single wavelength was employed, wood blocks painted gray were used as secondary standards. The secondary standards offered certain technical advantages: the difference between the sample and standard readings was greatly reduced, and the readings of the animals after injection were spread over a relatively wider portion of the galvanometer scale.

Statistical analyses were performed by accepted methods (Snedecor, '56; Bliss and Calhoun, '54).

Theoretical aspects

The most important factor in amphibian color change is the position of the melanosomes in the dermal melanophore cells (Noble, '54). After hypophysectomy, the melanosomes aggregate centripetally (con centration) near the nucleus of the melano phore cell, mostly underneath the guano phores. Upon injection of melanophor hormone, the melanosomes migrate centri fugally (dispersion) in the melanophores covering the sides and eventually nearl all the external surface of the guano phores. Enlarged photomicrographs o the dorsum of an hypophysectomized *Hyl cinerea* are shown in figure 1, before an after injection of a large dose of melano phore hormone. In the living frog, smal collections of melanosomes can be see with the dissecting microscope after hy pophysectomy; after a supramaximal dos of melanophore hormone, small, gree areas of the skin unobscured by the me lanosomes, are still visible.

The question of the relative importanc of melanophore hormone, other hormones nervous control, or direct response to ligh cannot be examined here; in our experi ence, healthy, uninjected, hypophysectom ized *Hyla* as well as *Rana* show only mino fluctuations in reflectance, while melano phore hormone injections are followed b major changes.

The physical model of this color chang would seem to be that of a completel opaque layer consisting of a two-colorant system: a mixture of nearly non-light scattering, large, opaque particles (me lanosomes) and a (light-scattering) turbi

[3] Purchased from the Munsell Color Company Inc., 10 East Franklin Street, Baltimore 2 Maryland.

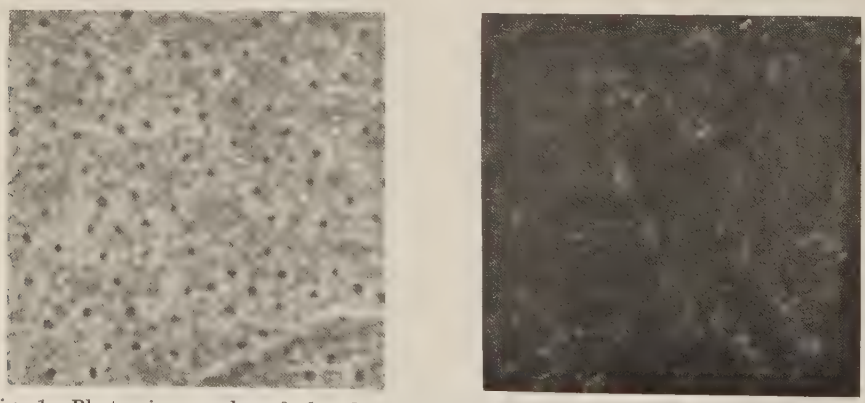

Fig. 1 Photomicrographs of the dorsi of hypophysectomized *Hyla cinerea*, uninjected (left) and after a supramaximal dose of melanophore hormone (right). Green filter.

medium. Under these circumstances, the directional reflectance value (R) obtained can be considered to be the reflectivity (R_∞) of the colorant layer.[4] At a given wavelength, the following equation, will give the ratio, K/S, of the absorption coefficient to the scattering coefficient of the layer:

$$\frac{(1-R_\infty)^2}{2R_\infty} = \frac{K}{S} \qquad (1)$$

The optical signature of the color of this layer would be shown by a plot of $\log_{10} (1 - R_\infty)^2/2R_\infty$ against the wavelength (Judd, '52).

As the melanosomes, under influence of melanophore hormone, migrate and begin to obscure the green of the medium, the reflectivity of the layer falls, reaching a minimum with a maximum dose. The additive function of reflectivity at each wavelength should be reflectivity, itself, and the laws of color mixture by averaging should apply. Thus, at each wavelength, the reflectivity of the mixture would be equal to the sum of the relative proportions of the pigments in the layer multiplied by their respective reflectivities, or,

$$R_\infty = f_b R_b + f_a R_a, \qquad (2)$$

where f_b and f_a are the proportions of melanosomes and of the light-scattering (green or non-black) pigment medium in the mixture, and $f_b + f_a = 1$. R_b and R_a are their respective reflectivities. A method for estimating R_b and R_a will be illustrated below. Since R_∞ at each dose level of a dose-response curve can be determined experimentally, the proportion (f_b) of melanosomes in the layer may then be related to the dose of melanophore hormone.

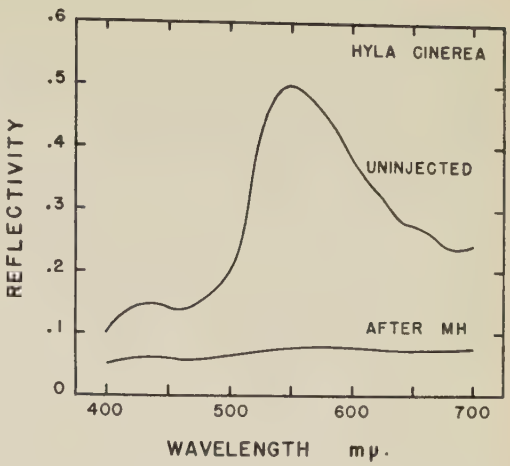

Fig. 2 "Fingerprint" reflectance spectra of the dorsi of hypophysectomized *Hyla cinerea* before and after a supramaximal dose of melanophore hormone.

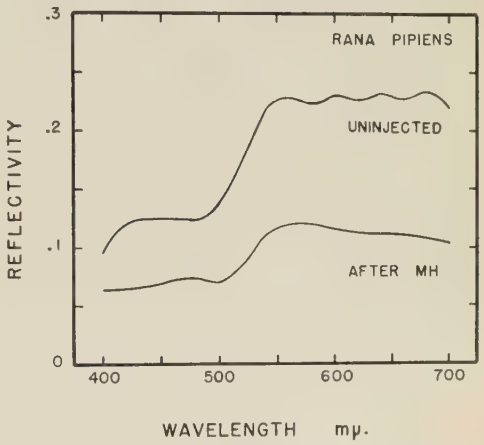

Fig. 3 "Fingerprint" reflectance spectra of the dorsi of hypophysectomized *Rana pipiens* (between fixed pigment spots) before and after a supramaximal dose of melanophore hormone.

RESULTS AND DISCUSSION

I. The reflectance spectra

The reflectance of a number of specimens of hypophysectomized *Hyla cinerea* and *Rana pipiens* was obtained at intervals in the visible spectrum. Typical examples of "fingerprint" reflectance spectra are shown in figures 2 and 3. The curves are drawn in a slightly idealized manner after averaging a number of readings. After injection of supramaximal doses of melanophore hormone, curves were obtained by moving quickly from point to point after the reflectance had reached its nadir. Although no great accuracy can be claimed for the post-injection curves, the general tendency towards a flattening at all wavelengths can be observed. (It would be desirable to confirm this impression by fol-

[4] In a previous publication (Teague and Patton, '59), we have analyzed the reflectance response as log 1/R, or reflection density, read on the absorbance scale of the instrument. Although this transformation is feasible as a measure of a biologic response, it does not yield the additional information obtained by the methods of this report.

lowing the change in reflectance at various wavelengths after small doses or by instantaneous recordings at varying times after a large dose, but instrumental limitations have precluded such observations.) Assuming that the flattening of the entire reflectance spectrum occurs at a constant rate, the fractional (or per cent) change in reflectance as a function of melanophore hormone dose will be the same at any wavelength between 400 and 700 mμ; actually, we have used the response at 550 with *Hyla* and at about 660 with *Rana* to take technical advantage of the greater galvanometer scale deflection offered at these wavelengths. The flattening of the reflectance spectrum at all wavelengths is more pronounced in *Hyla* than in *Rana*.

Transforming the reflectance of the uninjected hypophysectomized specimens to $(1 - R)^2/2R$ yields the curves of figure 4. The optical signatures are seen in figure 5, in which the log of this expression is plotted against the wavelength.

II. The dose-response relationship

A. The time-course of the response. Sufficient data were obtained on one 4.7 gm specimen of *Hyla* for a complete analysis (table 1). Five dilutions of Posterior Pituitary Solution were prepared in multiples of 2.5, the weakest solution having a concentration of 0.2 mU per dose of 0.1

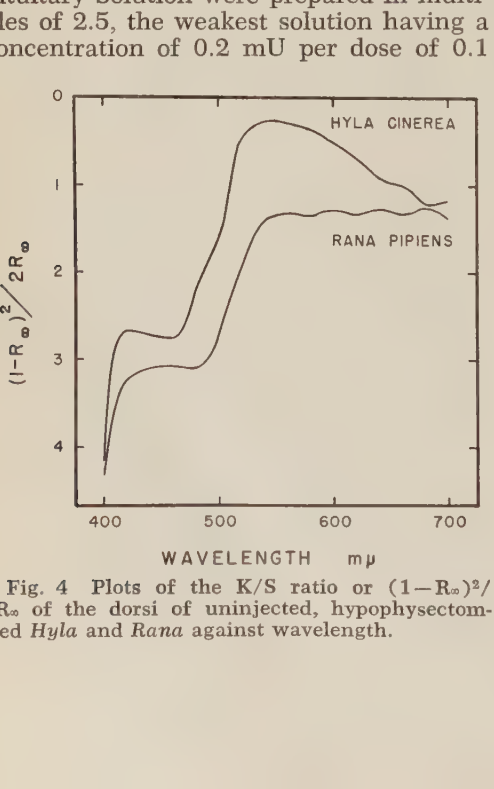

Fig. 4 Plots of the K/S ratio or $(1-R_\infty)^2/2R_\infty$ of the dorsi of uninjected, hypophysectomized *Hyla* and *Rana* against wavelength.

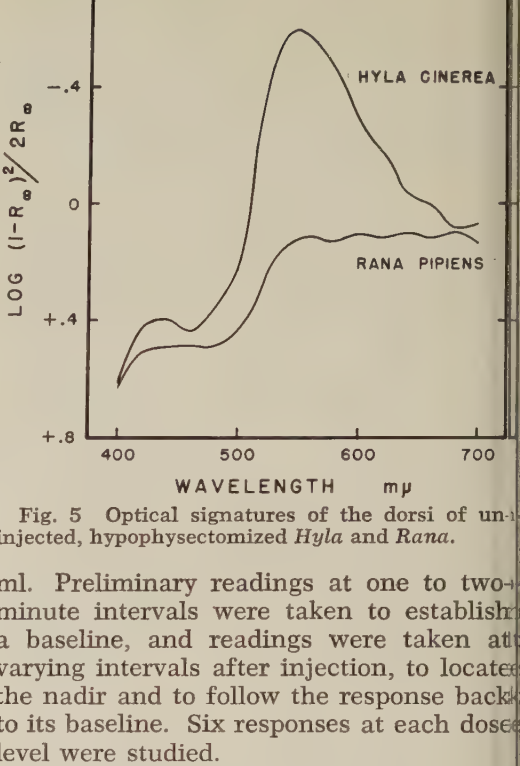

Fig. 5 Optical signatures of the dorsi of uninjected, hypophysectomized *Hyla* and *Rana*.

ml. Preliminary readings at one to two-minute intervals were taken to establish a baseline, and readings were taken at varying intervals after injection, to locate the nadir and to follow the response back to its baseline. Six responses at each dose level were studied.

For plotting, all responses have been subtracted from unity to give $(1 - R)$ values which visually rise instead of fall. Composites of the response at the 5 dose levels plotted against time, adjusted to a common baseline and slightly idealized, are presented in figure 6. It will be observed that the response parameter chosen may be the peak of the curve, the difference between the baseline and the peak, the area under the curve, or the duration of the response; all are functions of the dose, increasing monotonically from a minimum to a maximum value as the dose is increased. The sigmoid relationship of the duration of the response to log dose is shown in figure 7. It might be noted in passing that response duration has been proposed as an assay parameter with respect to frog web melanophores (Calloway, McCormack and Singh, '42); this study indicates the possibility of this approach with respect to reflectance, but a smaller error is found using other parameters.

TABLE 1

Dose-response reflectance data in one Hyla cinerea

Dose in mU, U.S.P. Post. Pit.									
0.2		0.5		1.25		3.125		7.8125	
Base	Peak	Base	Peak	Base	Peak	Base	Peak	Base	Peak
Reflectance readings as (1—R)									
0.641	0.689	0.631	0.750	0.642	0.884	0.703	0.920	0.650	0.925
0.653	0.687	0.657	0.803	0.634	0.875	0.655	0.897	0.709	0.923
0.715	0.733	0.680	0.812	0.676	0.872	0.673	0.918	0.673	0.927
0.655	0.699	0.640	0.810	0.689	0.896	0.678	0.914	0.643	0.924
0.713	0.713	0.708	0.813	0.666	0.870	0.652	0.906	0.675	0.913
0.664	0.691	0.675	0.832	0.659	0.903	0.619	0.906	0.733	0.930
Area under response curve as R·min.									
0.1		6.3		9.7		23.3		13.5	
1.3		6.5		13.8		13.0		29.3	
0.5		2.7		11.8		18.8		28.8	
0.7		5.7		21.0		18.1		40.0	
1.0		6.8		9.5		21.8		23.1	
1.5		5.0		16.7		30.3		26.5	

B. The peak response as 1 — R. The mean baseline (1 — R) was 0.6687 with a standard error of 0.0051 (30 cases). The readings at the peaks of the responses are given in table 1. Values recorded are the means of 4 individual observations around the peak. The response plotted against the natural dose is seen in figure 8, and the sigmoid plot against log dose is shown in figure 9. It is evident from figure 9 that an assay may be planned on the basis of responses at the first three dose levels. An analysis of the regression of the response expressed at ΔR is given in table 2; ΔR is the difference between the individual baselines and the peaks. In addition, ΔR has been corrected for baseline readings by covariance.

C. The area response. Each of the 30 responses were plotted against time after injection, and smooth curves drawn by eye through the points; the areas under the curves were measured with a planimeter and converted to the product unit, reflectance × minutes (R·min). The data are presented in table 1. The sigmoid relationship of this response parameter to log dose of melanophore hormone after a

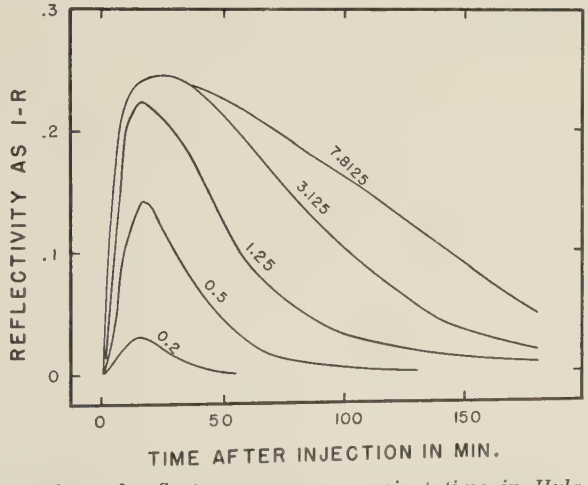

Fig. 6 Composite plots of reflectance response against time in *Hyla cinerea* at 5 dose levels, in mU U.S.P. Posterior Pituitary.

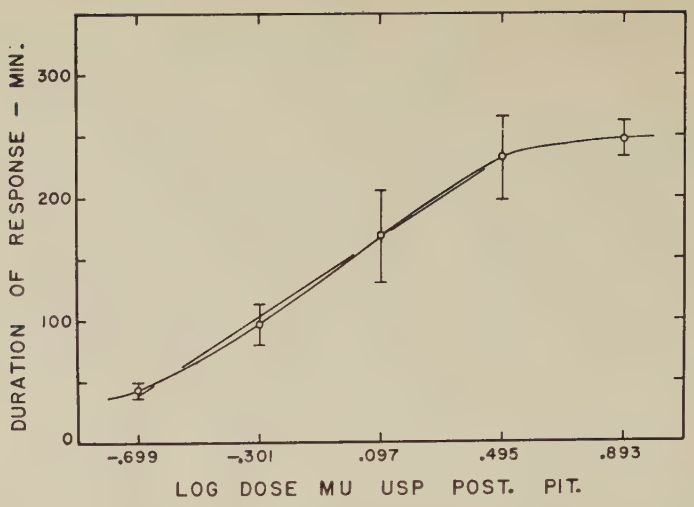

Fig. 7 Sigmoid plot of reflectance response duration in *Hyla cinerea* against log dose melanophore hormone. Bars indicate ± 1 standard error of the mean. The interrupted straight line is drawn to fit the equation, $Y = 135.7 + 32.10X$, describing the regression at the lower 4 dose levels.

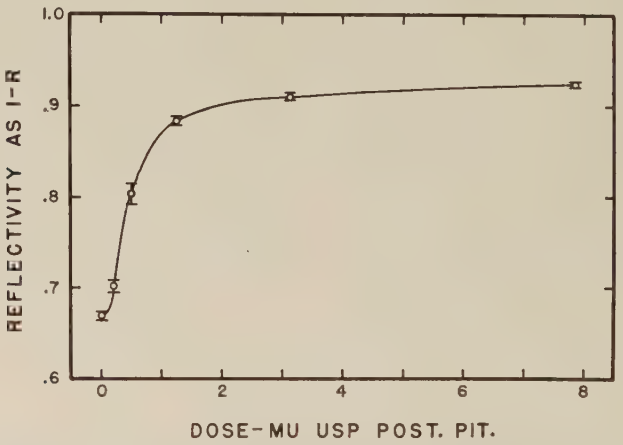

Fig. 8 Plot of the peak reflectance response in *Hyla cinerea* against melanophore hormone dose. Bars indicate ± 1 standard error of the mean.

square root transformation is seen in figure 10. As with the peak response, it is obvious that the latter can be used as an assay at the first three dose levels, but a detailed analysis will not be presented here. An assay on the basis of area would require too much time and effort for the slightly increased information obtained as compared with a peak response assay.

Results comparable with the data of section II were obtained with *Rana pipiens*.

III. Calculation of the relation to melanophore hormone of the proportion of melanosomes in the external (optical) layer of the skin

The preceding sections contain an empirical, practical treatment of the data, laying the foundation for a biological assay of melanophore hormone. At this point, by making certain assumptions, we shall attempt to relate the dose of melano-

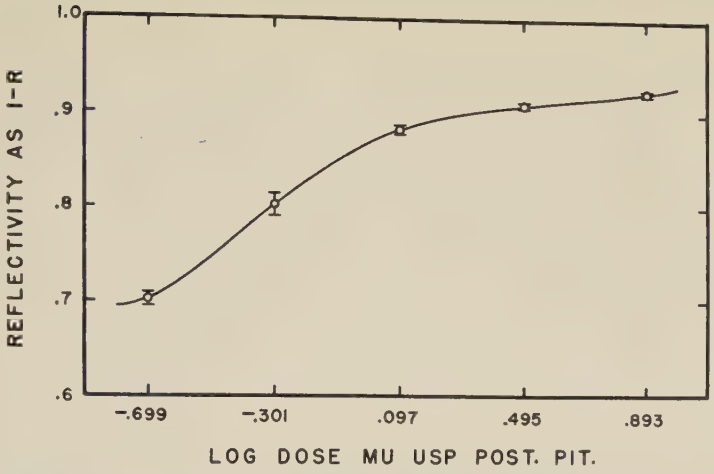

Fig. 9 Sigmoid plot of the peak reflectance response in *Hyla cinerea* against log dose of melanophore hormone. The curve approaches the mean baseline ($Y = 0.6687$) at the left and approaches a maximum at the right. A linear regression line fitting the equation, $Y = 0.8428 + 0.1770X$, would describe the response at the first three dose levels.

TABLE 2

Analysis of variance for peak response as ΔR at the first three dose levels. Analysis of covariance adjusted for baselines as R

Source of variation	df	SS	MS	F	P
Linear regression, adjusted	1	0.105209	0.105209	382.34	< 0.01
Quadratic term, adjusted	1	0.000543	0.000543	1.97	NS
Between doses, adjusted	2	0.105752	0.052876	192.16	< 0.01
Within doses (adjusted error)	14	0.003852	0.000275		
Total for covariance	16	0.109604			
Reduction of error by covariance	1	0.003127	0.003127	11.37	< 0.01
Within doses (unadjusted error)	15	0.006980	0.000465		
Linear regression, unadjusted	1	0.112714	0.112714	242.23	< 0.01
Deviations from regression	1	0.000650	0.000650	1.40	NS
Between doses, unadjusted	2	0.113364	0.056682	121.82	< 0.01
Total, unadjusted	17	0.120344	$\lambda = 0.070$	Bartlett's $\chi^2 = 0.45$	

Corrected regression: $Y = 0.200498 + 0.235298X$ Variance of slope = 0.0001448

phore hormone to the underlying biological process of melanosome dispersion.

Referring back to figures 2 and 3 and to section I, if we may assume that the fractional change in reflectance at every wavelength is equal, this equality may be expressed by the equation,

$$\frac{R_1 - R_2}{R_1 - R_b} = \frac{\Delta R}{R_1 - R_b} = k, \qquad (3)$$

where R_1 is the reflectance before injection, R_2 is the reading after injection, ΔR is their difference, R_b is the reflectivity of the melanosomes, and k is a constant. R_b is thus the theoretical minimum reflectance which would be reached at every wavelength, as f_b is increased to 1. It can be evaluated by solving equation 3 for ΔR:

$$\Delta R = -kR_b + kR_1. \qquad (4)$$

A plot of ΔR as a function of R_1 gives values for k and R_b. The slope of the resulting line will be equal to k and the R_1-intercept (the value of R_1 when $\Delta R = 0$) will be equal to R_b. The constants can be obtained with an accuracy which may be established statistically by treating equation 4 as a model I regression (Snedecor, '56) to which a least squares line may be fitted. The plots and equations are shown in figures 11 and 12, using data on figures 2 and 3 respectively. For the *Hyla cinerea*, R_b was found to be 0.057, giving a K/S

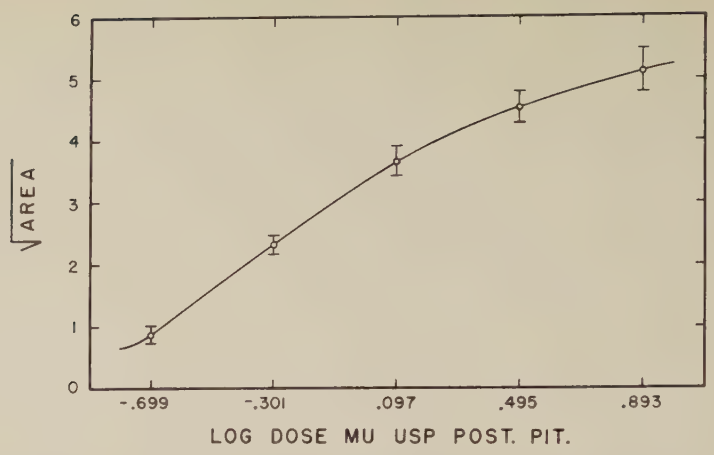

Fig. 10 Sigmoid plot of the relation of the response, measured as area (in R·min.) under the reflectance curve, after square root transformation, to log dose in *Hyla cinerea*.

ratio by equation 1 of 7.9. The R_b for the specimen of *Rana pipiens* was 0.034, K/S = 14.

It has been noted above that melanosomes are still visible in the uninjected, hypophysectomized frog dorsum. Consequently, the baseline reflectance (R_1) must be larger than the non-black pigment reflectivity (R_a), and at the baseline, f_b must be greater than zero. In order to evaluate R_a, we have examined microscopically the dorsi of the frogs. From these observations and from a study of photomicrographs, such as those shown in figure 1, we have estimated in *Hyla cinerea* the proportion of melanosomes of the skin color mixture as 0.05 before injection. The proportion in *Rana pipiens* seemed more variable, and for this reason R_a for *Rana pipiens* was not calculated.

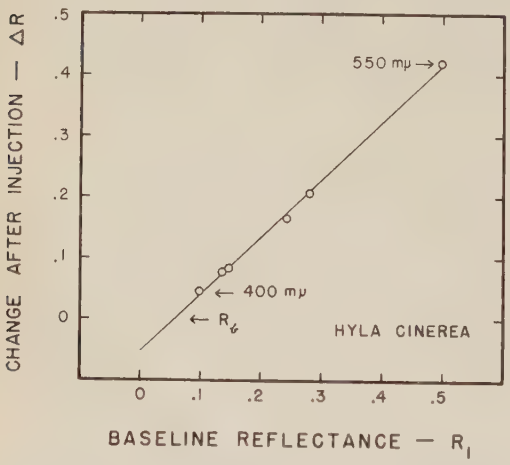

Fig. 11 Plot of data of figure 2 to indicate the graphic method of evaluating R_b in *Hyla cinerea*. The line is fitted by least squares to the equation, $\Delta R = -0.05321 + 0.9396\ R_1$. The R_1-intercept yields $R_b = 0.05663$. The wavelengths at which two of the points were obtained are indicated.

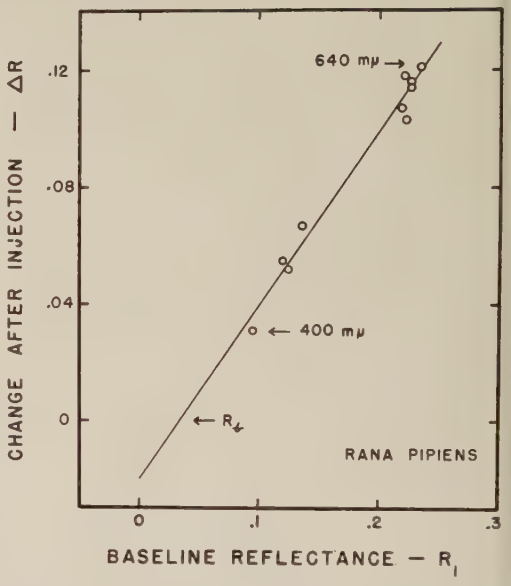

Fig. 12 Plot of data of figure 3 (*Rana pipiens*) in the manner of figure 11. The equation of the regression is: $\Delta R = -0.0204 + 0.5958\ R_1$; $R_b = 0.0342$. The wavelengths of two points are indicated.

Substituting 0.05 for f_b in equation 2, we obtained at each wavelength observed:

$$R_1 = (0.05)R_b + (1 - 0.05)R_a \quad (5)$$

whence the R_a at each wavelength may be obtained. Furthermore, the K/S ratio of the non-black pigment may be then calculated, using equation 1. Here, we will calculate R_a only at the wavelength used for assays. For *Hyla cinerea* at 550 mμ, the mean baseline reflectance was 0.3313. Substituting in equation 5:

$$0.3313 = (0.05)(0.057) + (0.95)R_a, \quad (6)$$

which yields an R_a of 0.35, K/S = 0.62.

The proportion of melanosomes at each dose level is obtained from the following equation, derived by solving equation 2 for f_b:

$$f_b = \frac{R_a - R_\infty}{R_a - R_b}. \quad (7)$$

Since equation 7 is essentially a statistical coding procedure for the peak $(1 - R_\infty)$ values of table 1, through algebraic subtraction of a constant followed by division by a constant, the mean f_b is immediately obtained by:

$$\bar{f}_b = \frac{R_a - \Sigma R_\infty/n}{R_a - R_b}, \quad (8)$$

and the sum of squares for f_b, by:

$$\Sigma d_{f_b}^2 = \frac{\Sigma d_{R_\infty}^2}{(R_a - R_b)^2} = \frac{\Sigma R_\infty^2 - (\Sigma R_\infty)^2/n}{(R_a - R_b)^2}, \quad (9)$$

where d^2 indicates squared deviations from the mean.

The calculations are summarized in table 3 and a plot of the relationship of $\bar{f}_b$ to log dose melanophore hormone is shown in figure 13.

If the kinetics of melanophore hormone activity obeyed a mass action law in combining with specific receptors on melanophores, as suggested by Rigler and Holzbauer ('53), then one might expect a logit transformation of either R_∞ or f_b to be linear to log dose (Reed and Berkson, '29). This is not the case, for after logit transformation a poor fit to a straight line was found. As a matter of fact, neither does the logit transformation of Rigler's own data fit a straight line very well. The problem will be considered further in a subsequent publication.

SUMMARY

The dermal melanophore response to melanophore hormone in *Hyla cinerea* and *Rana pipiens* has been studied after hypophysectomy by means of spectrophotometric directional reflectance. The reflectance spectrum of the dorsum in each was recorded and the reflectance response to U.S.P. Posterior Pituitary in *Hyla cinerea* was analyzed. From a consideration of the optical properties of the skin, the reflectivities of the melanosomes and of the green pigment medium of *Hyla cinerea* at 550 mμ were estimated and the effect of the hormone on the proportion of melanosomes in the optical layer of the skin was determined.

ACKNOWLEDGMENTS

The authors are grateful to Mrs. Patrick Jones, Mrs. Robert Lokey, Mrs. Paul Stabler and Mr. Dow Bozeman for technical assistance; to Dr. R. L. Manning of Beck-

TABLE 3

Relation to dose of melanophore hormone of the mean calculated proportion ($\bar{f}_b$) of melanosomes in the optical layer of Hyla skin

Dose post. pit.	Mean reflectivity	$R_a - \bar{R}_\infty$	$\bar{f}_b = (R_a - \bar{R}_\infty)/(R_a - R_b)$	Standard error of $\bar{f}_b$
mU	$\bar{R}_\infty$			
	$(0.345757 = R_a)$	0	0	
0	0.331300	0.014457	0.0500	
0.2	0.298000	0.047757	0.1652	0.0253
0.5	0.196000	0.149090	0.5157	0.0393
1.25	0.116000	0.229090	0.7923	0.0192
3.125	0.089830	0.255924	0.8852	0.0123
7.8125	0.076300	0.269424	0.9318	0.0082
	$(0.056627 = R_b)$	0.289130	1.0000	

$(R_a - R_b = 0.289130)$.

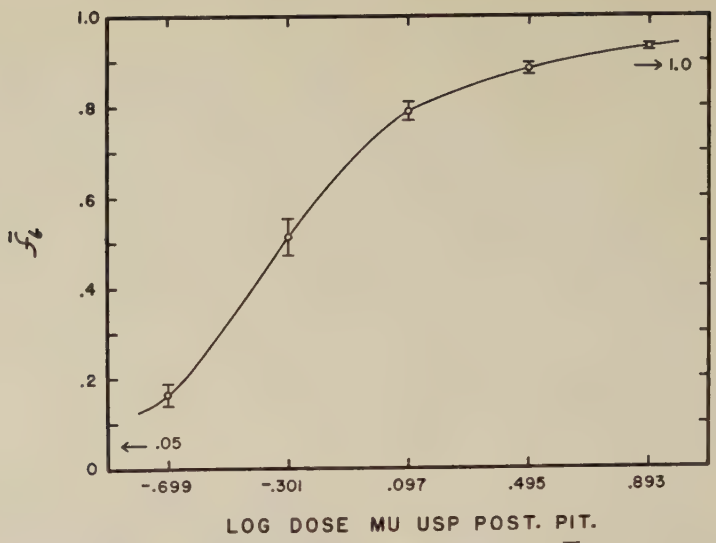

Fig. 13 Sigmoid plot of the relation of the mean proportion ($\bar{f}_b$) of melanosomes in the optical layer of the dorsi of *Hyla cinerea* to the log dose of melanophore hormone. Bars indicate ± 1 standard error of the mean. Arrows indicate maximum and minimum approached by $\bar{f}_b$.

man Scientific Instruments Division and to Dr. E. Scott Barr of the Department of Physics, University of Alabama, for advice in the conduct of this investigation.

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An Effect of Heavy Water on the Phase and Period of the Circadian Rhythm in Euglena

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Euglena, like several other single celled microorganisms, has a temperature compensated circadian rhythm. The phototactic response of *Euglena* is controlled by this rhythmic system (Bruce and Pittendrigh, '56, '58) and the cells characteristically respond to a light signal phototactically during the day time but less so, or not at all, during the night and a rhythmic alternation in the character of the phototactic responses will continue, with approximately a 24 hour period, in the absence of entraining light-dark or temperature cycles. As with other circadian rhythms the period of this rhythm changes very little with temperature. Between 20 and 33°C the period changes only from 24 hours to 23¼ hours. At 16.7° the period lengthens to about 26 hours but the phototactic responses decrease and below this temperature no rhythm has been demonstrated.

Essentially nothing is known about the physical-chemical nature of the mechanism of circadian rhythms and speculations about the mechanism have been handicapped by the failure of investigators to find chemical effects which might provide clues for further investigation. The writers have done a number of experiments designed to look for phase-shifts or period changes of the *Euglena* circadian rhythm by modifications in the physical-chemical environment. This paper reports results of preliminary experiments which demonstrate that both phase-shifts and period changes can be accomplished with deuterium oxide.

Before describing the heavy water experiments we would like to briefly review other experiments which we and other workers have done using drugs and chemicals in an effort to learn more about the nature of circadian rhythms.

Bühnemann ('55) could find no effect on the period or phase of the sporulation rhythm in *Oedogonium* using NaCN; 2-4 dinitrophenol; Na_2HAsO_4; NaF; quinine; $CuSO_4$; cocaine; β-indoleacetic acid; ATP; and riboflavin. Likewise Ball and Dyke ('56) using the inhibitors indole-3-acetic acid and 2-4 dichlorophenoxyacetic acid, found no phase shift or period change in the growth rate rhythm of the *Avena* coleoptile. Bünning ('56) used the rhythm of leaf motion in *Phaseolus* to investigate the effects of 2-4 dinitrophenol, acridine orange, ether, chloroform, colchicine, and phenylurethane. Only with the last two inhibitors was there any suggestion of an effect on the rhythm and Bünning interpreted his results with the mitotic inhibitors colchicine and phenylurethane as indicating a slowing down of the clock. Other workers, Wilkins ('59), Hastings ('60), using other organisms, have not detected any lengthening of the period of circadian rhythms in the presence of these inhibitors. Hastings ('60) has reported other recent attempts to shift the phase of the rhythm of luminescence in the dinoflagellate *Gonyaulax* by short-time (12 hours) exposure to chemicals, drugs, and inhibitors of various types. Both Bünning and Bühnemann have reported that pH changes are without effect on the rhythm.

Speculations regarding possible localization of the clock within the single cell have centered on the nucleus. Circadian rhythms of cell division occur both in protists and in metazoa (Sweeney and Hastings '58) but there is little evidence to suggest that this is anything more than just another rhythmic activity of the cell

which is controlled by the clock. Bünning and Schöne-Schneiderhöhn ('57) have investigated nuclear volume changes in non-dividing plant cells and have interpreted these speculatively in terms of structural changes within the nucleus. Ehret ('57) has investigated the phase-shifting of the rhythm in Paramecium using far-ultraviolet radiation. Although the effects are complicated by three superimposing effects there are indications that far-ultraviolet is a more efficient phase-shifting signal than visible light both with respect to intensity and duration of the signal. In Ehrets' system there are indications that the phase-shifts are photoreversible and it would be desirable to have further information on this important result. There are also some interesting results regarding methods of synchronization of plant and mammalian cell divisions. Sachs, Bretz and Lang ('59) have shown that cell division in the plants *Samolus* and *Hyoscyamus* may be synchronized by the time of application of gibberelic acid to a phase which is quite independent of the light cycle, and that these synchronized cells divide at approximately 24-hour intervals. The temptation to view this result as having possible significance for the biological clock is based on the approximate 24-hour period between cell divisions, a feature which it shares with mammalian tissue culture. The interest in the system lies however not only in the fact that the period is approximately 24 hours but also in the demonstration that it requires only a trigger to synchronize the cells and by the same token we consider it significant that a very brief (relative to the generation time) cold shock will synchronize cell division in mammalian tissue culture (Newton and Wildy, '59).

The writers have looked for effects on the period or phase of the rhythm of phototaxis in *Euglena* using a number of inhibitors and chemicals. We have failed to find any effects using the respiratory inhibitor KCN or the mitotic inhibitor phenylurethane. We have looked for effects using the adenine growth factor analogue 2-6-diamino-purine sulfate, the pyrimidine and nucleic acid analogue 2-amino-4-methyl-pyrimidine as well as the purines and pyrimidines adenine, guanine, thymine, cytosine, and uracil and the compounds adenosine and deoxyadenosine. In addition we have looked for effects using the growth factors gibberelic acid and kinetin. We have changed the pH of the medium between the limits 3.5 and 7.1 and have found no consistent effect. Experiments in which we have attempted to shift phase with ultra-violet light have been negative but inconclusive because of difficulties encountered with our particular assay technique. We have grown cells in a number of different media and if the cells show any rhythm at all it is always with a period close to 24 hours.

MATERIALS AND METHODS

Euglena gracilis (z strain) was grown in the unsupplemented simple medium described by Hutner, Bach and Ross ('56, table III). Most of the cultures were grown in screw cap vials at 22°C with a light cycle of 12 hours of light (approximately 10,000 lux of fluorescent plus incandescent) and 12 hours of dark. Ordinary water cultures were generally grown for two or three weeks before testing the rhythm of phototactic response. Attempts to grow fully deuterated cultures have so far not been successful but several presumably partially deuterated cultures have been grown. We have attempted to adapt *Euglena* to successively higher concentrations of heavy water as suggested by Katz et al. ('58). Cultures adapt readily to 20% D_2O in three or 4 weeks and when transferred to about 45% deuterium for another month they had grown sufficiently to be tested for phototactic response. Attempts to adapt to higher D_2O concentrations under these growth conditions have resulted in some growth but not yet sufficient for measurements to be made of the phototactic rhythm. The fact that higher concentrations of D_2O are not toxic is demonstrated by observations which we have made which indicate motility and viability (but very slow growth) in a culture left for two months in media with an initial D_2O concentration of 75% and in another left for 6 months in media with an initial D_2O concentration of 84%. There is of course some exchange of hydrogen for deuterium from the atmosphere in these conditions and in the long time intervals

involved here this will have resulted in some dilution of the deuterium content. The exchange of hydrogen for deuterium from the atmosphere was probably relatively more important in the change of the isotopic composition of the growth media of two cultures which were grown for two months in small carrell flasks in media of an initial composition of 95% D_2O.

When cultures are tested for the rhythm of phototactic response they are transferred to small carrell flasks and placed in the recording appparatus. A general description of the method of recording has been previously described (Bruce and Pittendrigh, '56) but the actual equipment has been redesigned and will be described in a later publication.

RESULTS

Unpublished experiments by the writers have demonstrated that the phase of the phototactic rhythm in *Euglena* is not changed if the cells are centrifuged and resuspended in new media of the same or different composition. We have also shown that the phase of the rhythms of two cultures which have been previously established so that they are 12 hours different from one another are not changed if the cells are centrifuged and resuspended in the supernatant of "in-phase" or "out-of-phase" cultures. There thus seems to be no direct effect of the interaction of the cell with its fluid environment on the rhythm of the cell. The adjustment of the cell to osmotic shocks, pH changes, etc. is apparently accomplished without any gross effect on its rhythm. These observations are pertinent to a consideration of the effects on the rhythm of replacing the media of H_2O-grown cells with D_2O media.

If H_2O-grown *Euglena* are put in D_2O-minimal media they immediately become non-motile and no phototactic response is observed at least for two or three days. This inactivity is reversible by transfer back to H_2O minimal media and it is thus possible to investigate the phase relationship of the phototactic rhythm of the cells before and after exposure of the cells to D_2O. Three experiments of this type have been done and the result of one such experiment is illustrated in the left-hand side of figure 1. Clearly, the phase of the resultant rhythm is shifted. The phase of the shifted rhythm seems to be correlated with the time of transfer of the culture from D_2O back to H_2O and this aspect is illustrated in the right-hand part of the figure. The circled points indicate the time of minimum phototactic response and the dashed arrows indicate the time during which the cells are in D_2O. The upper part of the figure includes two replicates one of which is illustrated at the left. The details of this type of phase-shifting have not been worked out and this is an aspect of the investigation which requires further study. The phase of control cultures (suspended in H_2O instead of D_2O) is not shifted in this type of experiment.

We have done several experiments in which the free-running-period of the rhythm of cells which have been adapted to D_2O by long term growth has been measured. Figure 2 illustrates the results of 4 experiments of this type and it is evident that the period length is increased by adapting to D_2O. The results indicated with curves A and B were obtained with two cultures which had grown for over two months in small carrell flasks in media with an initial D_2O concentration of 95% and curve C is a second run (two months later) of the culture represented by curve B. The culture represented by curve D was grown for one month in a screw cap vial in media of an initial D_2O content of 45% after the culture was initially adapted for three weeks in 20% D_2O. We have not observed any lengthening of the period of cultures adapted to 20% deuterium.

Cultures adapted to D_2O must be readapted to H_2O as Katz et al. ('58) have shown to be generally the case with algae which have been adapted to heavy water. The period of the rhythms of two cultures which we have adapted by growth from D_2O back to H_2O is in both cases close to 24 hours.

DISCUSSION

There have been quite a few recent studies concerning biological effects of deuterium oxide and a number of investigators, especially Katz and co-workers ('60) have shown that a considerable number of microorganisms may be successfully cultured in high concentrations of heavy water. It has in fact been demonstrated that in some

of these organisms the hydrogen may be essentially completely replaced with deuterium. Such completely deuterated cells are generally quite normal although there may be morphological differences and the growth rates are generally smaller. It has also been demonstrated that cells which are in the process of adapting to deuterium (or deuterated cells which are in the process of adapting to hydrogen) may show evidence of gross abnormality. These deleterious effects of isotopic substitution are however not toxicity effects and they are generally completely reversible.

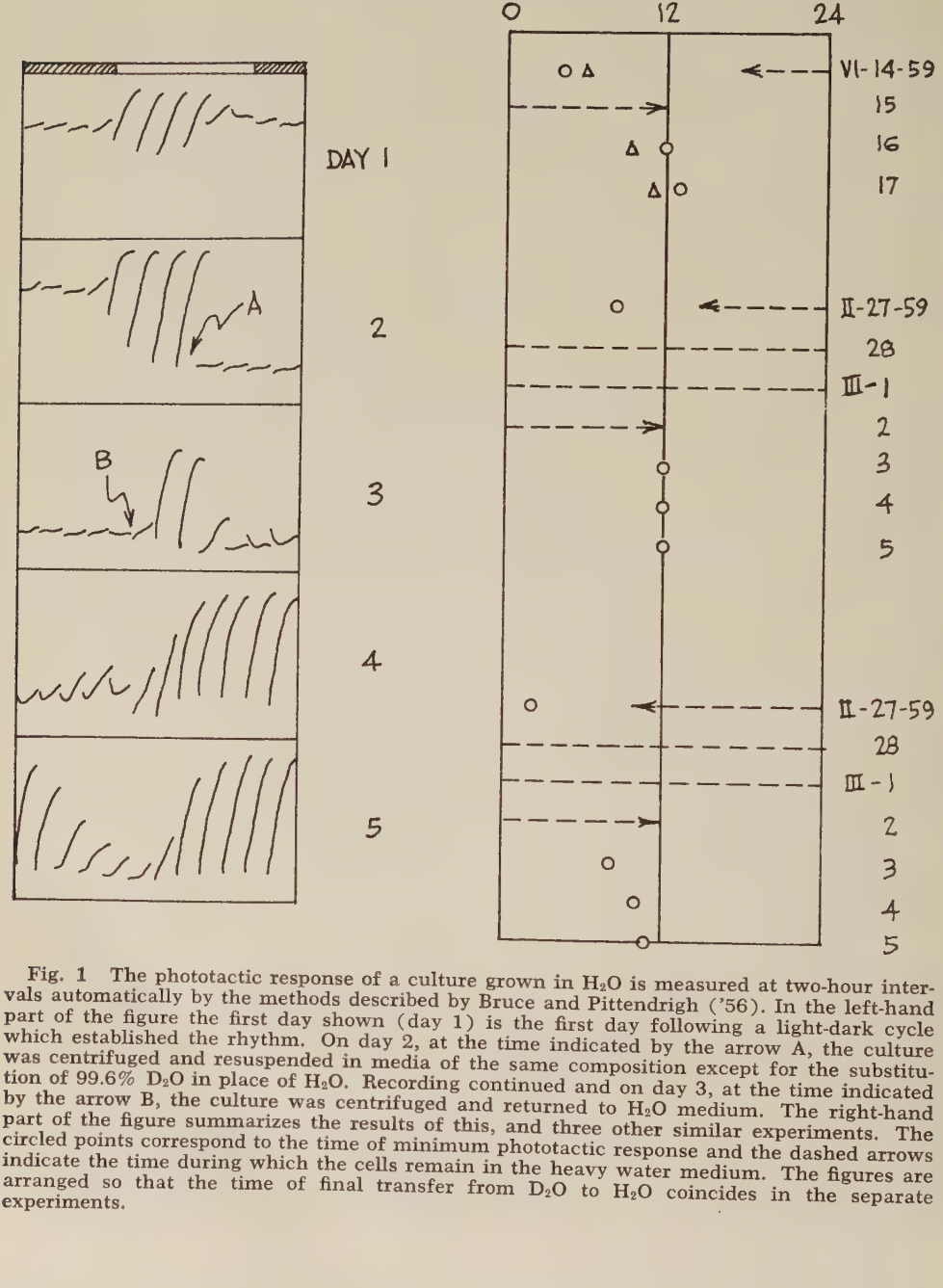

Fig. 1 The phototactic response of a culture grown in H_2O is measured at two-hour intervals automatically by the methods described by Bruce and Pittendrigh ('56). In the left-hand part of the figure the first day shown (day 1) is the first day following a light-dark cycle which established the rhythm. On day 2, at the time indicated by the arrow A, the culture was centrifuged and resuspended in media of the same composition except for the substitution of 99.6% D_2O in place of H_2O. Recording continued and on day 3, at the time indicated by the arrow B, the culture was centrifuged and returned to H_2O medium. The right-hand part of the figure summarizes the results of this, and three other similar experiments. The circled points correspond to the time of minimum phototactic response and the dashed arrows indicate the time during which the cells remain in the heavy water medium. The figures are arranged so that the time of final transfer from D_2O to H_2O coincides in the separate experiments.

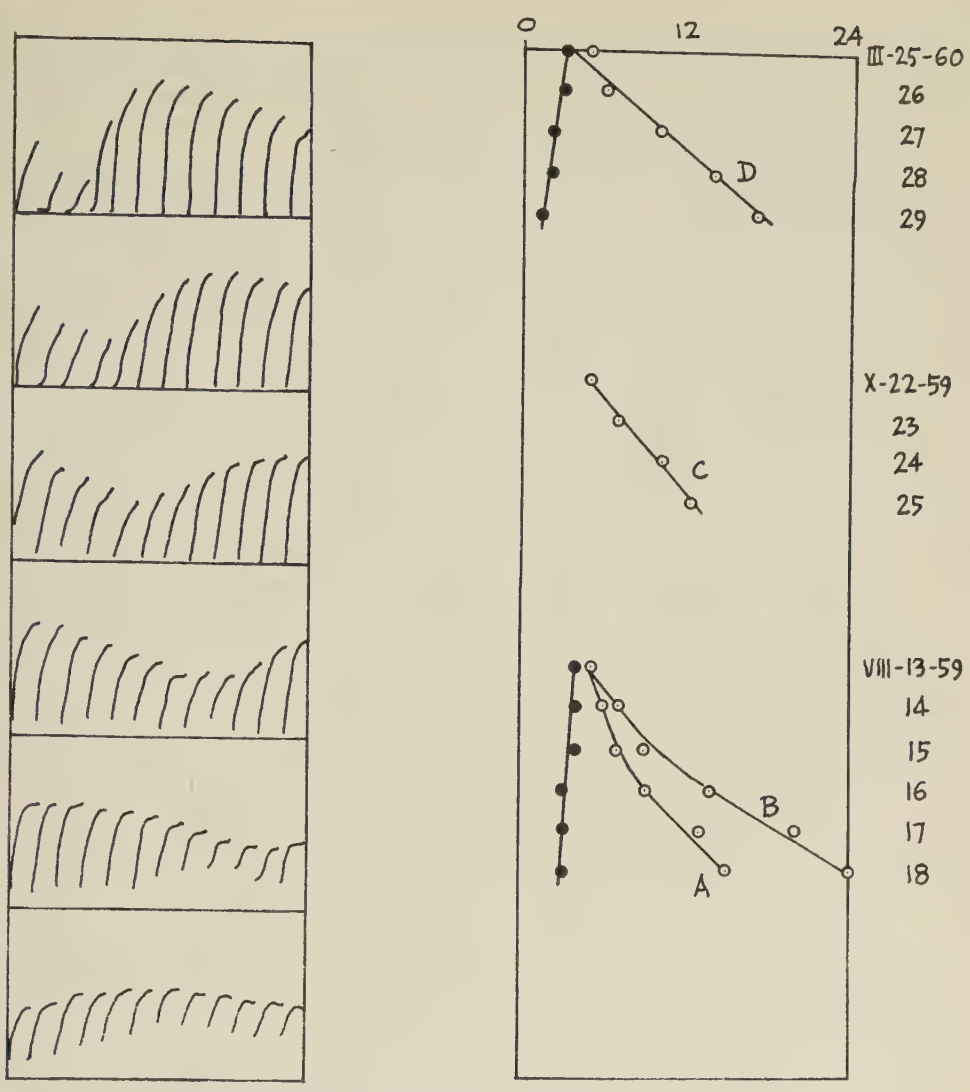

Fig. 2 The figure illustrates the lengthening of the period length of the circadian rhythm in *Euglena* cultures which have been adapted to heavy water. The left-hand part of the figure illustrates the record of the phototactic responses of a culture adapted to D_2O. The first 6 days following the establishment of a rhythm by a light cycle have been illustrated and one can see how the time of the minimum response shifts to a progressively later time of day and also that the rhythm rather quickly damps out. In the right hand part of the figure the summary results of this, and of other similar experiments are illustrated. The circled points correspond to times of minimum phototactic response on successive days. The open circles are cultures which have been adapted to D_2O whereas the filled circles correspond to control cultures grown in H_2O. Cultures A and B (B summarizes the results of the left-hand part of the figure) were grown separately and culture C is the same as B but tested about two months later. These are the cultures described in the text which were grown in carrell flasks for two months in media with an initial D_2O concentration of 95%. Culture D was adapted to grow in 45% D_2O. All experiments were carried out at 23°C. The period of the controls is between 23½ and 23¾ hours whereas the period of the adapted cultures ranges from 26½ to almost 28 hours.

Our own experiments may be discussed in relation to two different biological effects of heavy water. One is the immediate effect of replacing H_2O with D_2O. This, we have seen, does have some effect on the rhythm in *Euglena* whereas experiments by ourselves and others using quite a number of so-called toxic agents have failed to disclose any effects of these toxic agents on circadian rhythms. It is interesting in this connection to compare the observations of Gross and Spindel ('60) on mitotic arrest in *Arbacia* eggs suspended in D_2O. This effect, which is completely reversible, occurs at a stage when even the most drastic metabolic poisons have no effect in inhibiting mitosis. The *Euglena* experiments do not allow us to conclude that the rhythm is completely stopped when the cells are transferred from H_2O to D_2O, and then re-initiated when the cells are transferred back to H_2O. It may be that the phototactic responses are inhibited while the rhythm continues. Although there is some suggestion from figure 1 that the final phase of the rhythm is determined by the time of the final transfer to H_2O, there is also some suggestion that the old rhythm continues to exert its influence for the first day after the final transfer to ordinary water. Our exeriments are not sufficiently extensive to justify much speculation on this point but it is worth while to compare these results with those which are obtained in ordinary water when a rhythm is lost in continuous light and then re-initiated by transfer to the dark. In both cases a new rhythm is established but the phase relationship, relative to the transfer which initiated (or re-sets) the rhythm, is roughly 10 or 12 hours different in the two cases. It would be interesting to perform a reciprocal type of experiment in which a deuterated organism is transferred for a short time to ordinary water and then returned to heavy water, but we hope to culture completely deuterated cultures before trying this type of experiment. In speculating on the mechanism whereby the phase of the circadian rhythm is shifted by short-time exposure to D_2O one should include the possibility that it is mediated at the macromolecular level. Calvin, Hermans, and Scheraga ('59) have shown that under appropriate conditions some macromolecules may undergo a reversible phase transition from the helical to the randomly coiled form and that the transition temperature of this change may be significantly altered in D_2O as compared with H_2O.

The actual process of the adaptation of a microorganism to heavy water is not well understood and the partially deuterated organism is a more complex one than the completely deuterated one. We have not adapted a fully grown H_2O culture to D_2O without many generations of growth and we do not know whether this would be possible. There is some evidence from other organisms that deuterium has a mutagenic effect and in the absence of any demonstration that there is no such effect in *Euglena* we must allow of the possibility that the lengthened period of the rhythm of the adapted *Euglena* cultures is a genetic effect. However this is rather unlikely to be the explanation since the period lengthening of independently derived cultures is always about the same and since partially deuterated cultures re-adapted back to ordinary water again have a typical circadian period. Although there are no visible evidences of adaptation (motility, phototaxis, growth) during the first few days after transfer to heavy water it may be that in some sense the cells do adapt rather quickly and it might be of interest to follow other activities of cells which are in the process of adaptation. Our experiments have been with adapted cultures which have been too dilute to allow us to measure the extent to which deuterium was incorporated into the cells, therefore our evidence that they are partially deuterated is inferential and is based in part on the fact that a fairly long period of adaptation is required in adapting D_2O grown cells to H_2O.

There are of course a multitude of differences between the physical chemistry of ordinary water and deuterium oxide and we only wish to make two comments here regarding the possible relevance of these differences for the present problem. One possible cause of different biological response in heavy water and in ordinary water arises from the different physical properties of the two. One might expect that cells in heavy water at a given tem-

perature should perhaps behave like cells in ordinary water at a lower temperature. The period lengthening and rapid damping of the rhythm of adapted cultures is in some respects similar to the behavior of the rhythm in ordinary water at low temperatures and it would be useful to have more information on the effect of temperature on the period length in deuterated *Euglena*. It would also be especially useful to have information on the effect of deuteration on the period length of the rhythms of organisms in which the period length is decreased with a lowering of the temperature. The electrical conductivity of most salts is lowered in D_2O, and there are certainly electrochemical changes (pH or pD) arising from isotopic substitution. Experiments by ourselves and others, cited in the introduction, suggest however that sudden pH changes are without effect on the phase or period of the rhythm, although in our own experiments we have not directly followed the intracellular changes in pH.

The observations reported here are of a preliminary nature and further experiments are required to determine the probable mode of action of heavy water both in shifting the phase of the rhythm and in lengthening the period of the rhythm. Much has been learned in recent years about the biological effects of deuterium oxide and chemical studies of primary and secondary deuterium isotope effects are being actively studied. There are undoubtedly many complications in the interpretation of the biological effects of heavy water but we are reporting the present observations because they provide some encouragement for a sustained interest in searching for physical-chemical clues to the nature of the biological mechanism underlying circadian rhythms.

ACKNOWLEDGMENTS

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Electrophysiological Studies of a Non-Luminescent Form of the Dinoflagellate Noctiluca miliaris

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Can a protozoa produce action potentials of the all-or-none type when stimulated electrically? Is the boundary membrane between the vacuolar sap and the protoplasm capable of developing electrical responses? The experimental results of the present study indicate that an affirmative answer to one or both of the above questions seems inevitable.

Spontaneously recurring hyperpolarizing responses in a protozoa, *Noctiluca*, have been reported previously by Hisada ('57). The main part of his work is concerned with measurements of the resting potential in this protozoa with relatively large microelectrodes. The present investigations made on *Noctiluca* available in this country, show some discrepancies from the previous work done on the Japanese species. It was possible, in the present study to demonstrate with the aid of recent techniques, all-or-none action potentials in this protozoa in response to stimulation. Furthermore, investigations were made of impedance changes during activity, and of the influence of various agents in the external medium upon the action potential as well as of the voltage-current characteristics of the active and resting membranes of the cell. *Noctiluca miliaris* is a planktonic dinoflagellate which is well known for its luminescence. Studies on the luminous aspects of excitability in this organism have recently been made by Nicol ('58) and previously been reviewed by Harvey ('52). Its large size (0.2–1.5 mm in diameter) and its relative non-motility coupled with the recent success in mass-culturing it in the laboratory by Hastings and Sweeney ('57) make this organism one of the most easily handled protozoa for electrophysiological studies. The experience gained and the techniques developed through this work may very well serve as a good vantage point for such studies on other protozoa and small isolated single cells.

MATERIAL AND METHODS

Noctiluca miliaris, Suriray, kindly supplied to us by Dr. B. M. Sweeney of the Scripps Institution of Oceanography, La Jolla, California was used throughout this study. These specimens were collected from the Pacific Ocean, cultured in a medium of Erdschreiber-sea water containing *Dunaliella* as food organism in the laboratory of Dr. Sweeney until they were flown to Woods Hole. They kept well at a temperature of 10°C in a similar culture medium diluted by sea water. A number of protozoa were removed as they were needed, and the experiments were carried out at room temperature of 23–26°C and, unless otherwise stated, in sea water. Only those organisms fully distended and showing active movements of their muscular tentacles were used in these experiments. Parenthetically, this particular strain of *Noctiluca* were not luminescent. We were told that this was true even when they were freshly collected.

During the impalement by microelectrodes and the subsequent experimentation, the cell was held by a hydrostatic pressure in a specially designed chamber (fig. 1A). This chamber consisted of upper and lower pools of fluid, usually sea water, and had a connection through a small vertical cylindrical hole, 40 to 165 μ in diameter, in the glass plate forming the bottom of the upper pool. A cell could easily and securely be held on this hole by applying a hydrostatic pressure which could be readily changed by adjusting the fluid levels of the pools. Normally, 1 to 5 mm of fluid level difference was sufficient. Depending on the experiment, the size of the hole was changed by replacing the whole glass plate with one that had a proper sized hole. Two microelectrodes, having tip diameters of less than 0.5 μ and

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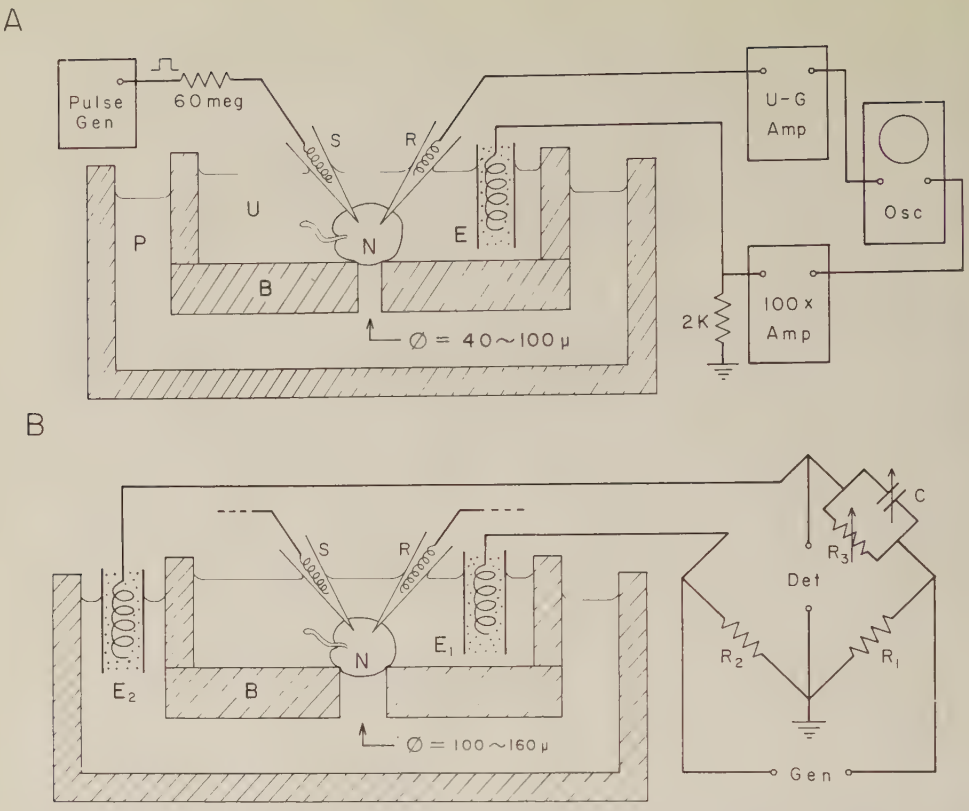

Fig. 1 Schematic representation of experimental setups. A, Arrangement used for recording electrical responses. The *Noctiluca* cell, N, under investigation was held on the hole in the replaceable bottom plate, B, by hydrostatic pressure caused by the difference in the water level between the upper pool, U, and the lower pool, P. The recording microelectrode, R, and the stimulating microelectrode, S, were inserted in the cell by means of micromanipulators. A large reference electrode, E, was connected to ground through 2 KΩ resistor across which the intensities of applied current were measured.

B, A.C. Wheatstone bridge used for detecting impedance changes during activity. The unknown arm of the bridge consisted of two large electrodes, E_1 and E_2, the cell, N, and the cylindrical hole having a diameter of 100 to 160 μ. E_1 and E_2 were of the Ag–AgCl (agar) type.

filled with 3 M KCl, were inserted into the cell with the aid of micromanipulators under direct visual control through a dissecting microscope.

The potential between the cell interior and the surrounding fluid was measured by connecting one of the impaling microelectrodes to a unity gain amplifier with a negative capacitative feedback (Bak, '58). Using a provision in this amplifier and test pulses supplied by a pulse generator (Tektronix, type 161), the resistance of the microelectrode was repeatedly measured at various frequencies. This resistance monitoring was carried out throughout the experiment for each sweep of the oscilloscope; during the critical period involving the impailment process the frequency was stepped up. The constant current pulse, mostly for the purpose of stimulation, was supplied by another pulse generator (Tektronix, type 161) through a 20 to 60 megohm series resistor and the second microelectrode. This current was measured with a low-level amplifier (Tektronix, type 122) as a voltage drop across a low value (2kΩ) resistor in the ground return from the large Ag-AgCl-agar electrode in the external fluid. A double beam oscillograph (Dumont, type 322-A) displayed the potential and current variations simultaneously, and

a Grass camera (model S4C) was employed for recording.

The impedance change was measured across the whole cell arrested on a relatively large hole (100 μ or larger in diameter) separating the two pools. Figure 1B shows an A.C. bridge in which one arm consists of the cell, the hole and two large electrodes in the surrounding pools in series. The two fixed resistive arms had a ratio of 1 to 100 (2 Ω and 200 Ω) and the fourth arm was made up of a variable resistor and a variable capacitor connected in parallel. Sinusoidal waves at the frequency of 2,000 cycles per second from an oscillator (General Radio Beat-Frequency Oscillator, type 1304A) were applied to the generator terminals through a symmetrical attenuation network, while the detection was made by one beam of the oscilloscope after an amplification through a Tektronix low-level amplifier (type 122). The current and potential microelectrodes were inserted into the cell as usual, and the potential variation was recorded concurrently with the impedance change through the remaining channel of the oscilloscope.

RESULTS

Resting potential

When both potential and current electrodes were confined in an intracellular space surrounded by a closed surface with a relatively high electric resistance, the current pulses applied through the current electrode should give rise to potential variations in the potential electrode, representing the IR drops across the surrounding resistance. Thus a successful impalement of a *Noctiluca* cell is signaled by the appearance of pulses on the potential trace of the oscilloscope without being accompanied by changes in the monitored resistance of the potential electrode before and after the penetration.

After each successful impalement of the cell by microelectrodes, careful observations were made throughout this study of the D.C. potential between the intracellular space occupied by both electrodes and the extracellular environment. In more than 200 successful impalements no significant resting potential was ever observed either on the D.C. coupled oscilloscope or on the electronic D.C. micro-voltmeter concurrently used with the oscilloscope. In many cases both electrodes were found to be clearly in the sap vacuole immediately after the impalement, and no D.C. potential existed. This latter case was also observed by Hisada. In time, the protoplasmic streams encircle the electrode tip, eventually forming a vacuole around the whole intracellular part of the electrode, and effectively and electrically speaking, push the electrode out of the cell. During any of these processes associated with the protoplasmic streaming, the D.C. potential remained constant almost indefinitely, even when the potential electrode was being "pushed out" by the aforementioned vacuole formation.

D.C. potential shifts, however, were observed whenever (especially during the course of impalement) there was a change in the microelectrode resistance which was measured by supplying a test pulse every one or two seconds. At times, though infrequently, there were random drifts of the whole system either to the positive or negative direction. These D.C. shifts could not be considered to be related to the existence of the resting potential, since when the electrode was very slowly moved from the cell to the external fluid, the shifted potential remained steady throughout the removal. Of course, if this removal process was accompanied by a change in the electrode resistance, potential shift may occur again.

Electric responses

When a sufficient current is passed across the cell surface in an inward direction (i.e., from the external fluid to the current electrode within), an electrical response can be elicited in *Noctiluca*. The potential variation in this electrical response has a polarity opposite to the normally observed electrical responses of most other cells, i.e., if *Noctiluca* were to have a normal resting potential like other cells, it responds with a spike towards the "hyperpolarizing" direction to a "hyperpolarizing" stimulating pulse. This response is "all-or-none" in character. It has a definite threshold for regenerative process and is preceded by a slowly rising subthreshold response which may subside or explode

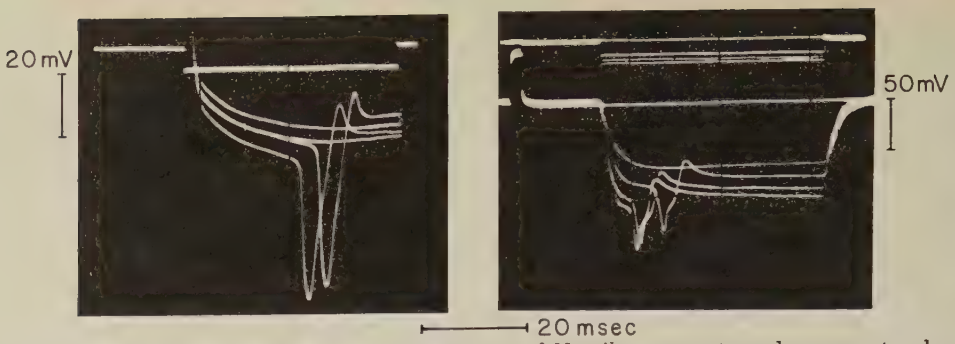

Fig. 2 Superposed records of electric responses of *Noctiluca* to rectangular current pulses of various intensities. A downward deflection of the potential (lower) trace represents a fall of the potential of the sap relative to that of the surrounding sea water. A downward deflection of the current (upper) trace indicates an inward flow of current through the layer of protoplasm. In the left-hand record the base-line of the potential trace is above the edge of the photograph. Note the gradual shortening in the latency with increasing pulse intensity. The resistance measurements of the potential electrode appear at the beginning of the potential traces in the right-hand record.

into a full response depending on the stimulus intensity. As can be seen in figure 2 which contains responses to just below, at and above threshold intensities of stimulus, the spike reaches to 55 mv from its point of inflection and on its return, it shoots past the steady state level giving a total deflection of about 72 mv. Judging from the direction and the magnitude of the potential variation, the responses which Hisada ('57) observed must have been of a similar kind although the slow sweep speed in his published material makes it almost impossible to determine the exact shape of the spike.

Such an "inside-negative" response could be demonstrated repeatedly with a remarkable reproducibility when identical stimulating pulses were given at favorable frequencies. Short pulses of the order of one msec. could elicit only single responses, whereas long pulses (from 10 to 50 msec.) might induce repetitive firing of up to several spikes when the specimen is healthy and has not previously been stimulated too many times. A typical record of such repetitive multiple responses to a single stimulus is shown in figure 3. The number of repetitive spikes triggered by a long single pulse gradually decreased as the specimen was being repeatedly stimulated, in the course of which the number of the spikes declined to one. Once thus the response was reduced to one per stimulus the repetitive firing might be recovered by giving the cell a rest period of about one minute.

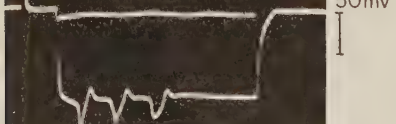

Fig. 3 Repetitive response of *Noctiluca* to a long pulse of constant current. Note the progressive decrease in the spike-amplitude and the prolongation in the spike-duration.

The time course of the response varied considerably depending on the experimental condition and the state of individual cells. Immediately after a successful impalement of a healthy cell, the spike duration was at its shortest, i.e., about 6.3 msec. excluding the overshoot. Repeated stimulation caused a gradual prolongation of the duration of successive responses, and the rate of prolongation became greater as the rate of repetition was increased. Repetitive stimulation is known to cause a prolongation of electric response duration in amphibian single nerve fibers (Spyropoulos, '56). Once prolonged, no complete reversal was observed in *Noctiluca*, although an extended rest period might shorten the duration of the spike to a small degree. A shortening of prolonged response period could also be brought about by a relatively fast rate of repetition of stimuli, e.g., faster than 0.5 per second, if either the previous stimulation rate was slower than this or a

rest period had preceded it. But such a shortening was only temporary and eventually, repeated stimuli, fast or slow in rate, would cause a further prolongation of the spike width.

Visual observations through a microscope revealed that on many occasions the movement of the muscular tentacle of *Noctiluca* immediately followed a stimulus and its ensuing electrical response. However, an electrical response was never observed to occur in correlation with a spontaneous tentacular movement.

Passage of current pulses in the outward direction did not bring about any electrical response while the current was passing. And in most cases, the termination of such a pulse also elicited no response, while in rare cases "break response" occurred after an outward current pulse.

Refractory period

By varying the interval between the two short stimulating pulses it was possible to measure the refractory period of the electrical response. When the pulse duration of 1 to 3 msec. was used, the shortest interval between the pulses that produced two responses ranged between 51 and 25 msec.

Effect of substitution of K, choline or sucrose for Na in the external medium

The sea water which normally surrounds the cell under study was replaced with various solutions consisting of different proportions of isotonic potassium chloride and normal sea water in order to lower the sodium concentrations at different levels. Replacement of all sodium but 10% of normal concentration in sea water showed no effect on the amplitude of the spike for as long as one hour. Sometimes the response disappeared under such conditions, but invariably the cell was then found to be dead or nearly dead judging from the softness of the cell body, crumpling of the protoplasmic substance or the failure to recover when the cell was returned to the normal sea water. Total replacement of sodium by potassium always resulted in a rapid and complete reduction of response amplitude followed by a cell death.

Artificial choline sea water or sucrose sea water containing no sodium was also employed to replace the normal sea water surrounding the cell in lowering the sodium concentration to various degrees. These experiments also showed full sized responses throughout the whole hour during which the cells were subjected to solutions with only 10% of its normal sodium. When no sodium was present in the external fluid, cells rapidly deteriorated to the point of death even though all other ions were available in these artificial sea water solutions.

Effect of chemicals

Cocaine, urethane, tetraethylammonium chloride (TEA) and nickel chloride dissolved in sea water were studied for their respective effects on the evoked response. Replacement of the normal sea water surrounding a normally responsive impaled cell with 5, 1 and 0.2% cocaine solutions resulted in a progressive and marked prolongation of the spike duration and eventually in a complete loss of the response. All this took place within one to two minutes from the time of solution change. 0.05% cocaine-sea water produced a similar type of widening of the spike but at a slower rate, while 0.005% solution produced a gradual prologation which began at about 2.5 min. of treatment and reached the maximum duration at about 8 min. Once it had reached this point, the response remained constant until the environment was changed to the normal sea water at about 20 min. A recovery at this time shortened the spike to as much as one-third of the maximum duration in 20 min. of washing with normal sea water.

Solutions having concentrations higher than 50% isotonic TEA in sea water killed the cells within a short time. A 20% isotonic TEA solution in sea water, however, produced a prolongation of the spike duration without much change in the amplitude of response for 16 min., after which it started to decline and reached the state of no response at 30 min. A mixture of one part of isotonic TEA and nine parts of normal sea water prolonged the spike to about 34 msec., but did not diminish the amplitude within 50 min. Washing with normal

sea water rapidly shortened the prolonged response duration.

A 3% urethane solution in sea water produced some prologation of the response duration but no decrease in the amplitude until the response suddenly disappeared at about 15 min. Cells examined at this time were found to be at the point of death. Higher concentrations of urethane produced cell deaths very promptly. Nickel ion which produces a marked prolongation of spike duration of vertebrate single fibers (Spyropoulos and Brady, '59) did not show any obvious effect on the *Noctiluca* response when introduced in the form of 10^{-2} N solution of nickel chloride in sea water.

Current voltage relations and the membrane resistance

A series of experiments was carried out to relate the intensity of the applied constant current and the resultant potential changes recorded across the cell membrane. Figure 4 shows a typical current-voltage relation obtained in this series. There is a clearly linear correlation between the current and the voltage as the former is gradually increased from zero in either direction. As the current reaches a certain threshold value in the inward direction there is a sudden increase in the peak value of the voltage; thereafter, the peak value of the voltage again increases in a linear fashion but at a decreased rate. The discontinuity in the current-voltage relation then represents the transition from an inactive state to an active one and the second straight line the increased conductivity in the active state. Plotting the values of steady state voltage level (instead of the peak voltage) against the current values also demonstrates a clear cut increase in conductivity in the active phase (broken heavy line in figure 4). This increase closely parallels the aforementioned change observed in the plotting of the peak values. The membrane resistance calculated from the slopes of such current-voltage plottings ranged from 1.1 to 1.8 $\times$ 10^3 ohm·cm^2 with an average of 1.4 $\times$ 10^3 ohm·cm^2 for the resting state. But at the peak of the active state it ranged from 0.58 to 0.72 $\times$ 10^3 ohm·cm^2 with an average of 0.65 $\times$ 10^3 ohm·cm^2. Apparently the conductivity of the cell membrane is increased by a factor of about 2 when the membrane is pushed to an active state. The effective e.m.f. of the active state, obtained by extrapolating the current-voltage curve in the active state to zero current, is

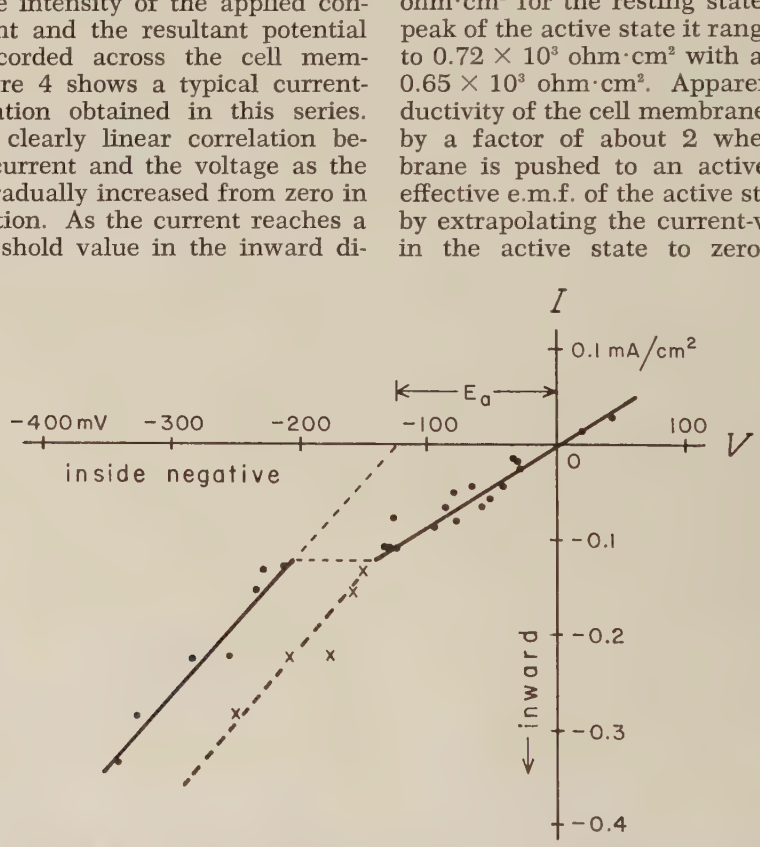

Fig. 4 Relation between the intensity of applied current (ordinate) and the shift in the intracellular potential (abscissa) caused by the current. The two values in the range of current stronger than the threshold value represent the potentials at the peak of activity (dots) and the potential levels in the subsequent steady state (crosses). E_a indicates the effective e.m.f. at the peak of activity.

shown as E_a in figure 4 and ranged from 115 to 137 mv with an average of 125 mv. There was no sudden break or non-linear change in the rate of voltage increase as the current was increased in the outward direction.

Membrane capacitance

Since the input time constant of the unity gain amplifier through an electrode resistance of 20 megohms can be adjusted to be less than 10 μsec., the slow potential variation caused by a rectangular current pulse can be attributed to a "capacitance" connected in parallel to the membrane resistance. The membrane capacitance, calculated from the oscillograph tracings and the membrane resistance previously calculated, showed the spread of 0.42 to 2.3 $\mu F/cm^2$ with a mean of 1.3 $\mu F/cm^2$. The above values were for the application of inward current. The time constant of the cell membrane for the outward current was the same, whereas that for the termination of the current pulse was measured to be slightly longer.

Impedance change

The current-voltage relationship measurements demonstrated a pronounced change in the impedance between the resting and the active states. Even though such static measurements establish the existence and the absolute magnitudes of the change, these do not yield the dynamic time course of the impedance variation in relation to the potential change during activity. Using a sensitive A.C. bridge circuit across the cell which separated the upper and the lower pools of the chamber, simultaneous recordings of the potential change and the bridge unbalance during activity was successfully attained. Figures thus obtained (fig. 5) show a direct correlation between the time courses of the action potential and of the bridge balance. The bridge unbalance begins to increase at the moment when the action potential is initiated and both the potential and the unbalance reach their peaks at about the same time. Apparently both also have very similar time courses during their recovery paths. The bridge unbalance observed here was ascertained to be toward the decreased impedance, i.e., increased conductance, by previously unbalancing the bridge in a known direction and observing the direction of balance change during the activity. The pattern is, then, a direct correlation in the time courses of the two wherein the impedance reaches its minimum at the peak of the action potential.

Reverse potential responses

A search was made for clues as to the possible origin of the action potential inasmuch as the action potential ordinarily

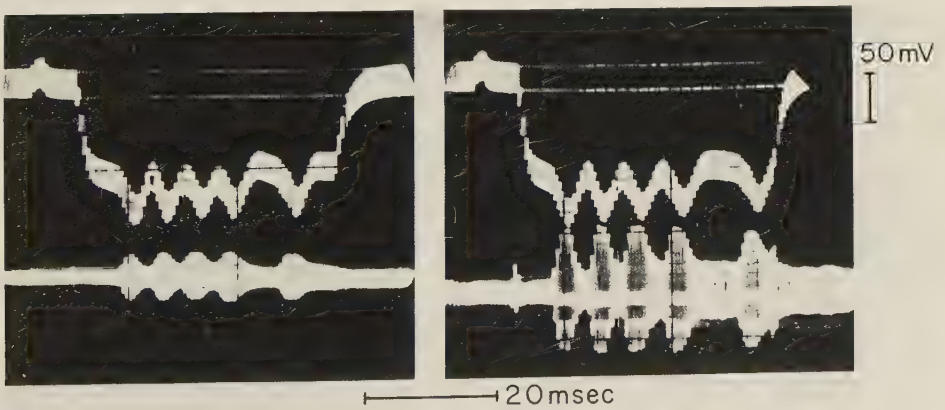

Fig. 5 Changes in the electric impedance of a single cell of *Noctiluca* associated with electric responses. The upper trace represents the intracellular potential on which the bridge A.C. is superposed. The lower trace shows bridge unbalances during activity. The bridge was balanced roughly for the impedance of the cell at rest. Note the simultaneity between the responses and the impedance loss.

recorded in *Noctiluca* was opposite to that of many other known cells and in view of the fact that the recording electrode was for the most part in the sap vacuole rather than in the protoplasm. This potential change may arise from either the outer membrane, the inner membrane facing the sap, or both. One effort in this direction, i.e., the introduction of the recording microelectrode into the protoplasm per se with the aid of a high power microscope, produced some interesting records. In those instances where the electrode was inserted very superficially or into the dense protoplasmic mass at the base of the tentacle, small potential changes took place in the reverse direction, i.e., the site of the microelectrode became more positive as measured from the imposed baseline caused by the inward current pulse. In other words, the direction of the response was occasionally similar to the conventional nerve, muscle or other excitable cells, i.e., in the "depolarizing" direction. Unlike the highly reproducible responses in the "hyperpolarizing" direction, the shapes of such "depolarizing" responses from *Noctiluca* were not repeatable from cell to cell. Within each individual cell, however, these responses not only showed reproducible shapes but were also all-or-none in nature and the latency shortened as the stimulus was gradually increased beyond the threshold intensity. Other attempts as, e.g., approaching the protoplasm with the microelectrode from the sap side or penetrating into the muscular tentacle, failed to produce any significant results.

DISCUSSION

These observations made in this study on the responses induced by applied current pulses suggest three distinct possibilities as to the site of their origin as well as some interesting and significant biological implications.

If the outer membrane were responsible for the production of these observed "hyperpolarizing" responses, *Noctiluca* then presents a bona fide case of reproducible all-or-none "response in the reversed directions" under normal environment. Alternatively, there is a possibility that the observed responses are produced at an intracellular membrane, i.e., the phase boundary between the protoplasm and the sap. It is evident that at least one or the other must actually be the case.

In the first possibility, the potential variation during activity is in the direction in which the cell interior becomes more negative during the spike with respect to its previous state, or according to the conventional description, the response is truly "hyperpolarizing." Such responses are not entirely unknown. For instance, this sort of response is known to occur reproducibly: in squid giant axon (Segal, '58; Tasaki, '59a) and in myelinated single nerve fibers of amphibians (Mueller, '58; Stämpfli, '58; Tasaki, '59a) both subjected to high external potassium concentrations; in frog dorsal root ganglion cells immersed in barium rich solutions (Tasaki, '59b); in Purkinje fibers of dog's heart under low temperature (Chang and Schmidt, '60); and although not strictly analogous, in secretory cells of the salivary gland (Lundberg, '55, '57). These, however, occur only under abnormal conditions or do not possess the all-or-none character. Against this first possibility, it should be pointed out that in *Noctiluca* the response amplitude remains unaltered while the external chemical environments are drastically changed.

If one assumes instead that the inner membrane facing the extensive sap vacuole is producing the response, one may consider the sap fluid of *Noctiluca* acting as the external space of the ordinary response situations in other cells, and the protoplasm, though it surrounds the sap, as the cell interior. Then the direction of the stimulating current and that of the response can be regarded as being similar to the situation in ordinary excitable cells; currents flowing from the protoplasm outward across the membrane elicit responses which will make the protoplasm more positive during the spike relative to the vacuolar sap. Also, in ordinary cases the impedance of the membrane decreases during activity, and so does the observed impedance change in this study. The insensitivity of the action potential to the external ion changes and to many of the chemical agents dissolved in the surrounding sea water may indicate that the mem-

brane responding might be well protected by the outer plasma membrane and the layer of protoplasm. It seems quite unlikely that the outermost pellicle of the cell would act as a barrier, especially to the smaller ions. Furthermore, Hisada ('57) reports that the potassium content of the sap is similar to that of the sea water. The increase in the concentration of potassium or decrease of sodium in the surrounding sea water would then be expected to have very little effect on the sap composition. These considerations point to the likely existence of the regenerative all-or-none response of the intracellular membrane between the sap vacuole and the protoplasm.

It is not inconceivable that both membranes may be involved in the production of the normally observed response. The occurrence of all-or-none "depolarizing" responses at a time when both electrodes are barely inside the cell as well as the difference in the shapes of responses before and after prolongation of the spike duration suggest such a possibility. This situation is similar to that which was postulated by Osterhout ('34) on the cell of *Nitella*. It might be pointed out in this connection that the observed time course of the potential difference across the cell caused by a rectangular current pulse is given by a simple logarithmic curve. This fact appears to mitigate against this third possibility.

Whenever a microelectrode with a diameter of 1.5 μ or larger was employed during this study, it was extremely difficult to impale a cell; furthermore, when the impalement was made, damage to the cell was so great that a proper sealing was rarely obtained. Hisada's measurements of resting potentials were made between the time of protoplasmic enclosure of the 2 μ tip and the vacuole formation around it. Continuous electrode resistance monitoring in the present study showed that after the tip was placed in the sap, the electrode became extremely unstable as soon as the protoplasm started to engulf the tip. Eventually it became impossible to measure the action potential when the vacuole formation was completed. It is therefore, very difficult to draw any conclusion from the D.C. potential level observations made without, as was presumably the case with Hisada, precise knowledge of the stability and the resistance of the electrode throughout the period.

It is noteworthy that the measurements of conductivity during activity by means of an A.C. bridge parallel the data obtained by an entirely different method, i.e., by measurements of V–I relations before and during activity. Both sets of data show a definite increase in the conductance during activity. Such was the case in squid axon (Cole and Curtis, '39) and frog single fiber (Tasaki and Mizuguchi, '48). The values of membrane resistance, 1.4×10^3 ohm·cm^2, and capacitance, 1.3 μF/cm^2 for *Noctiluca*, including the two membranes, compare favorably with those obtained from other organisms; for example, in squid the membrane resistance and capacitance are estimated to be 1×10^3 ohm·cm^2 and 1.1 μF/cm^2 respectively (Curtis and Cole, '39); in frog single node, 8–20 ohm·cm^2 and 3–7 μF/cm^2 (Tasaki, '55); in *Fundulus* eggs 3.4×10^3 ohm·cm^2 and 0.63 μF/cm^2 (Kao, '56); and in starfish eggs, 3.1×10^3 ohm·cm^2 and 0.5 μF/cm^2 (Tyler et al., '56).

The electrical responses observed in this study were usually the result of inward or anodal current pulses applied through the micro-electrode inserted within the cell. Break responses to cathodal or outward pulses which Hisada described briefly could only be obtained rarely and then with difficulty. The reasons why Hisada was not able to get the responses to inward current pulses and why there never was a specimen which gave spontaneous electrical responses associated with the spontaneous tentacular movement in this study are not clear. Since most of Hisada's published response records were based on such spontaneous phenomena, it may be possible that there are some differences between his *Noctiluca* specimens and ours. The luminescence is, e.g., one quality where our specimen differed from his. For these reasons, we have made attempts to obtain Japanese specimens, but thus far we have not been successful. A more detailed study (with a proper light intensity recording setup) of the relations between

the induced luminescent response and the electrical activity would seem a wonderful project for the future.

SUMMARY

1. A detailed study on the electrophysiological properties of *Noctiluca* has been carried out using hyper fine microelectrodes, a unity-gain amplifier, pulse generators and a dual-beam oscilloscope.

2. No significant resting potential was observed in more than 200 cells successfully impaled. The resistance of the recording electrode was continuously monitored before, during and after each impalement.

3. Electrical response which has a polarity opposite to the potential variations in the (hyperpolarizing) responses of most other cells, is elicited when a sufficient inward current flows across the cell surface. This response is all-or-none in character with a definite threshold.

4. Such electric response did not decrease its amplitude when all but 10% of the sodium normally present in the sea water was replaced with either potassium, choline or sucrose. Ten per cent isotonic TEA, 0.005% cocaine and 3% urethane solutions prolonged the response duration without decreasing its height.

5. Current voltage relations in *Noctiluca* show a linear relationship which exhibits a discontinuity and a change in its slope at the threshold current strength. An increase in conductivity associated with activity has also been recorded using an a.c. bridge circuit.

6. Membrane resistance and capacitance during the inactive state averaged 1.4×10^3 ohm·cm^2 and 1.3 μF/cm^2 respectively.

7. Alternative possibilities of whether such response is a true "hyperpolarizing" response or an intracellular response have been discussed.

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Metabolic Studies on Frog Skin Epithelium[1]

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"Active transport" across isolated living membranes was first described in frog skin (Huf, '35a, '35b, '36a). It was observed that isolated frog skin is able to move electrolytes (NaCl) and water from the epithelial side to the corium side of the skin in the absence of and even against a concentration gradient. Moreover, it was found that this transport of electrolytes and water was diminished when small amounts of enzyme inhibitors were added to the salt solutions bathing the isolated skin or when skins of frogs were used which were pretreated with enzyme poison. The relation between transport of electrolyte solutions across the skin and skin metabolism was, thus, strongly suggested. Since these early observations on active transport, frog skin has been often used in studies to elucidate the biophysical pumping mechanism which leads to salt accumulation on the corium side of frog skin. Ussing and Zerahn ('51) have shown that the skin acts directly on sodium ions, moving them in the inward direction; chloride ions follow passively in the electric field. In spite of the frequent choice of frog skin in studies on active ion transport there is a great lack of knowledge about metabolic reactions in epithelial cells of frog skin. A number of investigators have made measurements to find out how many sodium ions are transported for each molecule of oxygen consumed (Leaf and Renshaw, '56, '57a; Zerahn, '56a, '56b, '58; Huf et al., '57; Huf and Doss, '59). Extensive use has been made of enzyme inhibitors (Huf, '35, '36a; Huf et al., '57; Fuhrman, '52). The fact that a number of specific inhibitors such as iodoacetate, fluoroacetate, azide, dinitrophenol, depress the rate of active ion transport and oxygen consumption points to involvement of glycolytic reactions, the Krebs cycle, the oxidative chain and oxidative phosphorylation reactions in active ion transport. It seems, however, that unless more is known about each of these reactions in frog skin, the mechanism and the energetics of active sodium transport cannot be fully understood.

The experiments described in this paper were carried out with the following aims in mind. First, to provide for information on electrolyte composition and the metabolism of isolated skin epithelium, and secondly to apply conventional biochemical methods of investigation of cellular metabolism to homogenates of epithelial cells of frog skin. Brief reports of these studies have already been published (Skjelkvåle et al., '58, '59a, '59b).

METHODS

Studies on isolated skin epithelium. Large frogs (*Rana pipiens*) were killed by cutting the spine and pithing. The abdominal skin, the least glandular part of the skin, was cut out and cleaned of all adhering tissue. The skins were kept at 5°C in *Petri* dishes with 10 ml of a 4 mM $NaHCO_3$ solution, adjusted to pH 7. Eighteen to 20 hours later, the epithelium could easily be removed from the corium by scraping. Histological studies showed that a complete separation of the epithelium from the corium was achieved (fig. 1). The *Warburg* technique was used for measurements of oxygen uptake (Umbreit et al., '57). Epithelium of several abdominal skins was pooled and the pool was divided into several portions. Each portion corresponded to about one half of the epithelium of the abdominal skin of one frog. This was equal to 15–30 mg dry weight. The salt solution used had the following

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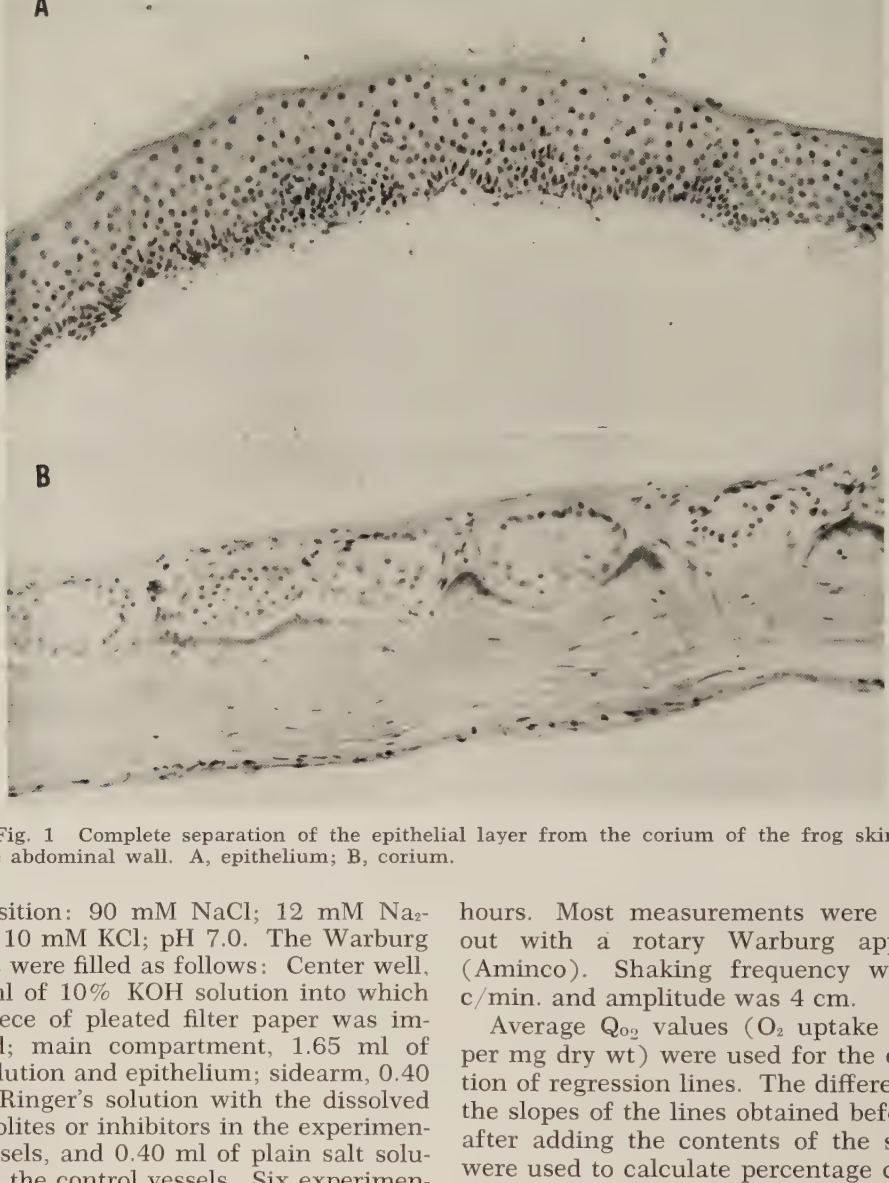

Fig. 1 Complete separation of the epithelial layer from the corium of the frog skin of the abdominal wall. A, epithelium; B, corium.

composition: 90 mM NaCl; 12 mM Na_2HPO_4; 10 mM KCl; pH 7.0. The Warburg vessels were filled as follows: Center well, 0.15 ml of 10% KOH solution into which one piece of pleated filter paper was immersed; main compartment, 1.65 ml of salt solution and epithelium; sidearm, 0.40 ml of Ringer's solution with the dissolved metabolites or inhibitors in the experimental vessels, and 0.40 ml of plain salt solution in the control vessels. Six experimental vessels were accompanied by 6 or more control vessels. All measurements were carried out in air at 20°C. Fifteen to 20 minutes were allowed for equilibration of temperature. Readings were taken for two hours at 30 minute intervals. The contents of the sidearm were then added to the main compartment and 15 minutes later the readings were continued for two hours. Most measurements were carrie out with a rotary Warburg apparatu (Aminco). Shaking frequency was 11 c/min. and amplitude was 4 cm.

Average Q_{O_2} values (O_2 uptake per hr per mg dry wt) were used for the calcula tion of regression lines. The differences i the slopes of the lines obtained before an after adding the contents of the sidearm were used to calculate percentage change in oxygen consumption. Bacterial growt in the Warburg flasks was not a matte of concern in 4- to 5-hour experiment (fig. 2e). It was observed, however, tha after many more hours of incubation th rate of O_2 consumption increased. This in crease could be prevented by the additio of penicillin and streptomycin to the sal solution in the vessels (fig. 2f).

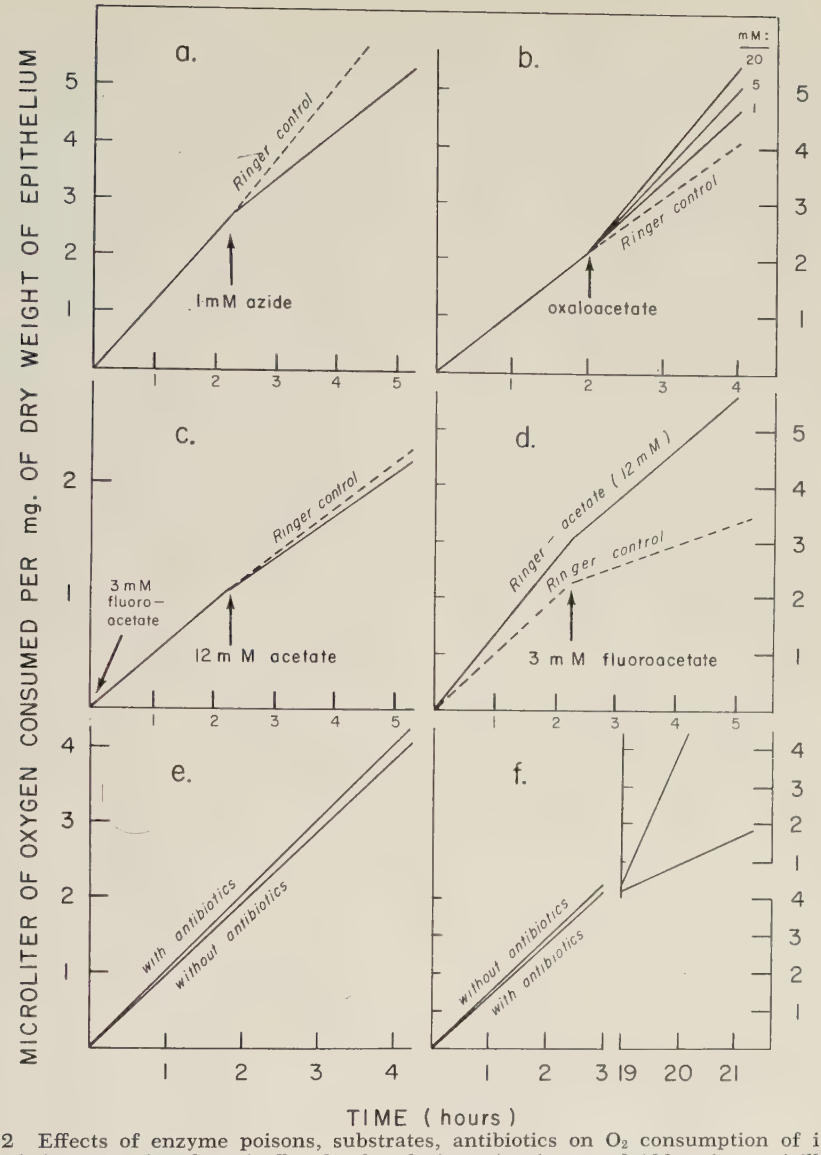

Fig. 2 Effects of enzyme poisons, substrates, antibiotics on O_2 consumption of isolated skin epithelium in phosphate buffered salt solution. A mixture of 100 units penicillin and 100 μg of streptomycin per ml of salt solution was used in experiments e and f. Shown are the calculated regression lines obtained from several experiments in each series (two in c and d, 5 and more in the other series).

A 25% and 50% increase in the NaCl concentration of the salt solution decreased the oxygen uptake by about 2% and 6% respectively. The differences were not significant. This point was tested because metabolites were used in the form of sodium salts.

Sodium and potassium content of epithelium, corium, and of whole skin were measured by flame photometry of a solution of the ash of the respective tissues (Huf, Wills and Arrighi, '55). Water content of the tissue was calculated from weighings of the samples before and after drying at 120°C for two hours.

Studies on epithelial homogenate. Frog skin epithelium is a rather tough tissue. It is much more difficult to obtain a satis-

factory homogenate of skin epithelium than it is of kidney or liver tissues which were used here for comparison in several studies. A microscopic examination of the epithelial homogenates which were used showed the presence of some intact cells, small sheets of epithelium composed of, maybe, several hundred cells, and abundance of particulate matter. After many failures the following procedures were adopted: 10 to 20 frogs were used in the preparation of homogenate for 12 Warburg vessels. The frogs were killed and the abdominal skin excised and cleaned of adhering tissue. The skins were placed in 50–75 ml of a solution of 4 mM $NaHCO_3$ and 2 mM di-sodium ethylenediamine tetracetate (Versene), pH 7. The solution containing the skin was placed in an ice bath and was magnetically stirred for one hour. After rinsing the skin with isotonic KCl and drying it between filter papers, the epithelium was removed by lightly scraping the surface of the skin. The scrapings were suspended in isotonic KCl and immediately centrifuged for three minutes at about 1000 rpm. The supernatant fluid was poured off and the remaining epithelium was dried between thick layers of filter paper, weighed and suspended in homogenizing medium to yield a 10% homogenate. Homogenization was carried out with small batches of epithelium. Before taking aliquots the combined homogenates were magnetically stirred while being cooled in ice. In this way samples of fairly uniform activity were obtained.

Media for homogenization. Ice cold isotonic KCl solution (154 mM) was used with success throughout most of the experiments. Novikoff's medium (Novikoff, '55) was used when attempts were made to fractionate the homogenate. Since these efforts, so far, have been unsuccessful no details will be reported. It was seen, however, that homogenates prepared with Novikoff's medium had about the same activity as those prepared with isotonic KCl. All media were 3 mM with respect to Versene and contained 0.1% of Antifoam B (Dow-Corning).

Homogenization was carried out in 50 ml thin walled, ice cooled tubes of the Potter-Elvehjem type. The pestle with cutting teeth was attached directly through a chuck to a heavy duty stirring motor with friction clutch. Homogenization at 2000 rpm (5000 rpm for the first few seconds) was carried out for periods of not more than one minute with as many strokes as possible. Intervals were allowed between these periods for cooling of the contents of the tube. This was continued until the pestle slid easily up and down the tube. The high speed of 2000 rpm, used here versus the more conventional speed of 800–1500 rpm was necessary to prevent sticking.

To establish some criterion for the tightness between tube and pestle, the tube was filled with 25 ml of distilled water and the pestle was pushed to the bottom of the tube. Experience has shown that the best tube-pestle combination was one in which it took about 15 seconds for the pestle to glide out of the water after releasing on the tube.

Oxygen consumption was measured with the Warburg apparatus (see above). The center well of the reaction vessels contained 0.2 ml of 10% KOH. Two ml of ice cold homogenate were mixed in the reaction vessel with 1 ml of ice cold reaction mixture (table 1). Preliminary experiments showed that the optimal pH was from 7.1–7.6 and the optimal temperature from 34 to 38°C. All experiments reported here were done at pH 7.1 to 7.2 and at 35°C.

Chemicals. All common chemicals were reagent grade. Double glass distilled water was used throughout this study. Substrates and other special chemicals were obtained from Sigma Chemical Company.

TABLE 1

Composition of reaction mixture containing 200 mg homogenized epithelium. The mixture is about 250 milliosmolar

Component	μmoles per 3 ml
Tris (hydroxymethyl)-aminomethane	60
KH_2PO_4	40
K-acetate	12
$MgCl_2$	12
ATP	3
Cytochrome c	0.03
Yeast concentrate	3 mg
Substrates as K-salts; (equimolar amounts of KCl in controls)	12
Versene	6

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RESULTS

A. Studies on intact epithelium

1. Electrolytes and water content: Q_{O_2}. Table 2 gives average data on electrolyte and water content, and on rate of O_2 consumption of isolated epithelium, corium and intact skin. The data on water, Na^+ and K^+ content of fresh skin check with results obtained in earlier work from this laboratory (Huf, Doss and Wills, '57).

Fifty to 60% of the Na^+ and K^+ was lost when skin was kept for 20 hours in 4 mM $NaHCO_3$ solution (see under methods). Both Na^+ and, especially, K^+ of the skin were found in higher concentrations in the epithelial layer than in the corium. It is known that the epithelium represents about one-sixth of the cross sectional area of preserved skin (Huf, '55). It will be noted from table 2 that the O_2 consumption of the corium was negligible as compared to O_2 consumption of the epithelium. Paired pieces of skins of several frogs were kept at 5°C in 4 mM $NaHCO_3$ solution for one and for 24 hours respectively. When the epithelium was removed and studied it was found that the average rate of O_2 consumption was the same in both cases (Q_{O_2} values of 1.53 and 1.56 respectively). A statistically insignificant 10% increase of O_2 uptake by epithelium was seen when the skins were pretreated in 4 mM $NaHCO_3$ and 10 mM KCl solution, to prevent losses of skin potassium (Huf, Wills and Arrighi, '55).

2. Factors influencing O_2 consumption. Figure 2 summarizes results of experiments in which the influence of several factors on oxygen consumption was investigated. Azide (1 mM) quite effectively depressed tissue respiration (fig. 2a), while oxaloacetate stimulated oxygen uptake (fig. 2b). Sodium acetate did not abolish the inhibitory effect of fluoroac-

TABLE 2

Water, electrolyte content of tissues; Q_{O_2} data[1]

	H_2O	Na^+	K^+	Q_{O_2}
	%	*μeq/gm dry tissue*		*μl/hr/mg dry tissue*
a. Intact skin, fresh	81.5	269	150	0.60
b. Intact skin, treated[2]	85.8	115	68	
c. Epithelium of treated skin	88.8	111	137	1.20
d. Corium of treated skin	73.0	62	29	negligible

[1] Average values calculated from (a) 12; (b) 4; (c) 7; (d) 8 experiments.
[2] Skin was kept for 20 hrs. at 5°C in 4 mM $NaHCO_3$ solution at pH 7.0.

TABLE 3

Influence of metabolites on respiration of isolated frog skin epithelium

Figures express percentage increase over controls (see under methods). Data are averages of results of 6 to 16 experimnts for each substrate concentration.

Substrates (Na-salts)	Substrate concentration, mM		
	1	5	20
Citrate	2	16	47
cis-Aconitate	9	20	40
dl-Allo-isocitrate	43	72	26
α-Ketoglutarate	5	24	38
Succinate	12	42	96
Fumarate	8	22	53
l-Malate	2	12	31
Oxaloacetate	27	55	65
Pyruvate	22	33	30
Pyruvate + oxaloacetate	—	52	58
l-Aspartate	9	28	31
l-Glutamate	12	39	40
β-Hydroxybutyrate	1	14	12

etate when this poison was added to the salt solution at zero time and the addition of acetate followed later (fig. 2c). If the acetate was added at zero time and then, at a later time, the system was poisoned with fluoroacetate, the protective effect of acetate was quite noticeable (fig. 2d; 60% inhibition in the absence of, and 30% inhibition in the presence of acetate). The significance of the results shown in figures 2e and 2f is explained under methods. More data on the influence of metabolites on the rate of respiration are given in table 3 (see also Huf, '36b). It will be noted that all the substrates which were tried stimulated tissue respiration. Some of them had their optimal effect at 5 mM concentration, others brought about further increase of O_2 uptake when given at 20 mM concentration. Succinate and ox-

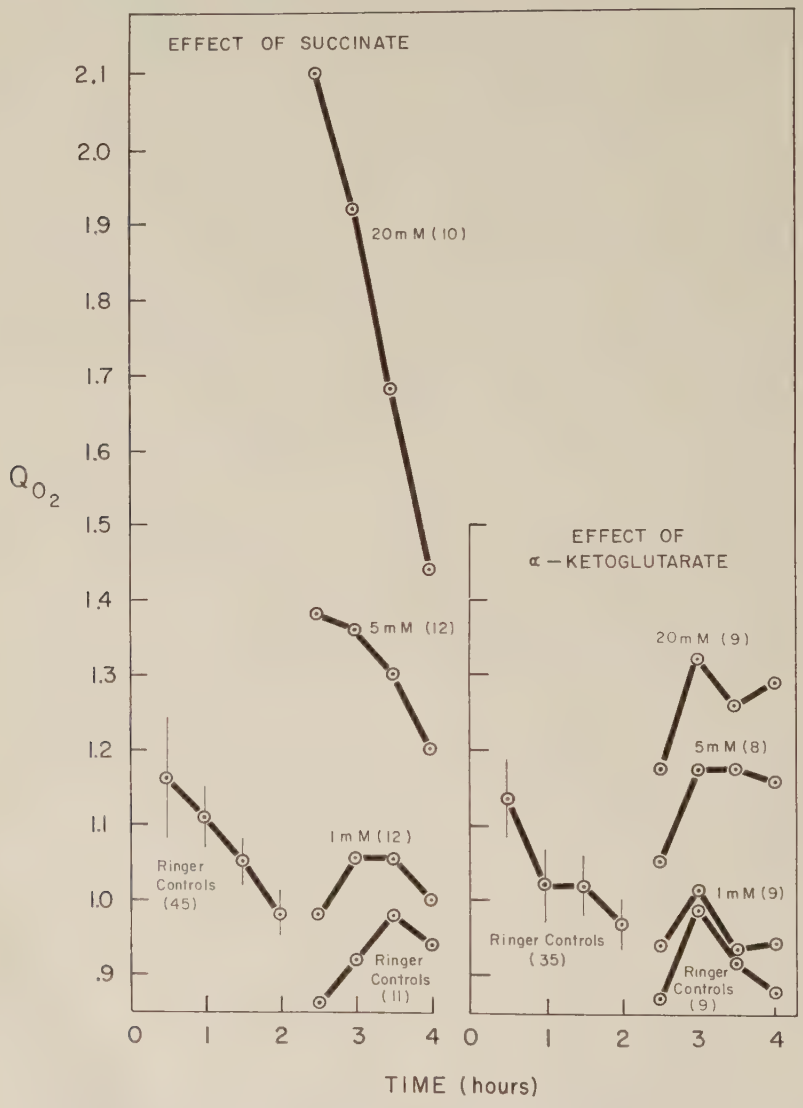

Fig. 3 Changes of Q_{O_2} values with time for skin epithelium kept in phosphate buffered salt solution (controls) and for epithelium in salt solution with succinate and α-ketoglutarate. Numbers of experiments are given in the illustration in parenthesis.

TABLE 4

Oxygen consumption of rat kidney homogenate (RKH), frog skin epithelial homogenate (FSEH), the latter Versene-treated and untreated, and mixtures of both homogenates

Composition of reaction mixture, see table 1. Substrates: 4 mM fumarate + 4 mM pyruvate. Temp. 35°C. Total O_2 consumption = endogenous O_2 consumption + net O_2 consumption, i.e., O_2 consumption resulting from metabolism of added substrates. Averages of two to 4 experiments.[1]

	1		2			3			4			5		
	Final versene conc. in Warburg flask, mM		0			0.33			0.33			1.0		
	System	Substrate	Tissue wet wt.	O_2 uptake Total	Net	Tissue wet wt.	O_2 uptake Total	Net	Tissue wet wt.	O_2 uptake Total	Net	Tissue wet wt.	O_2 uptake Total	Net
			mg	*µl/hr.*		*mg*	*µl/hr.*		*mg*	*µl/hr.*		*mg*	*µl/hr.*	
a	RKH	—	40	70		40	128		20	36		20	43	
		+		453	383		529	401		218	182		296	253
b	FSEH	—							200	67		100	15	
		+								181	114		83	68
c	a+b (calculated)	—								103			58	
		+								399	296		379	321
d	RKH+fresh FSEH	—	40+	144		40+	270		20+	233		20+	98	
	(measured)	+	200	486	342	200	663	393	200	467	234	100	455	357
e	RKH+ boiled FSEH	—	40+	87		40+	106							
		+	200	344	257	200	508	403						

[1] Results of other similar experiments are in agreement with the data shown in the table.

aloacetate were the most effective substrates while β-hydroxybutyrate was the least effective. It was noted that shortly after the addition of succinate, the stimulation of respiration was greater than it was after some time had passed. This observation is illustrated in figure 3 which shows the rapid decrease of the Q_{O_2} values with time. This was not the case with α-ketoglutarate (fig. 3) and other metabolites. These observations indicate that the method of comparing slopes of calculated regression lines to express quantitatively the effects of metabolites on respiration, as used here, has its limitations. The data presented in figure 2, therefore, describe only average effects.

B. Studies on epithelial homogenate

1. Effect of Versene. The ability of the homogenate to oxidize a mixture of 4 mM fumarate and 4 mM pyruvate was first investigated. It soon became apparent that frog skin epithelium contained some material which inhibited tissue respiration. Since the inhibitory factor was heat stable it was assumed to be a heavy metal and attempts were made to remove it with chelating agents. Diethyldithiocarbamate, a copper-specific chelating agent, failed to remove the inhibitory factor. Versene, which effectively can remove three valent iron, was therefore tried. Some results showing the inhibitory action of skin epithelial homogenate on rat kidney homogenate respiration and the effects of Versene are shown in table 4. Boiled skin epithelial homogenate acted as a stronger inhibitor than fresh skin epithelium homogenate (see "net" data, column 2). When skin epithelium was homogenized in the presence of Versene, partial and complete removal of the inhibitor of respiration was observed (columns 3 to 5, table 4). In fact, (see column 5) the combined kidney and Versen treated skin epithelial homogenate showed higher oxygen uptake than expected from the rates of oxygen consumption of either homogenate alone. This was the result of an increased endogenous respiration which was always seen when the mixture of the two tissue homogenates was used. Systematic studies have shown that no advantage was gained by the addition of Versene to the bicarbonated water in which the skins were kept preceding the removal of the epithelium. The optimal Versene concentration in the homogenizing medium for removal of the inhibitor was 3 mM. After removal of the inhibitor, preparations of fairly constant metabolic activity for periods of 1 to 2 hours were obtained.

2. Factors affecting the rate of respiration of epithelial homogenate. In all the experiments described below, substrate solutions of 4 mM fumarate and 4 mM pyruvate were used. If the reaction medium of Aisenberg and Potter ('55) contained 0.01 mM cytochrome c, a 6-fold increase of O_2 uptake, as compared to controls with no cytochrome c, was seen. Application of 0.1 mM cytochrome c concentration led to a further but rather small increase in the rate of O_2 consumption. Addition of 0.1% *yeast concentrate* to 0.01 mM cytochrome c doubled the rate of respiration as compared to controls with no yeast concentrate. Table 5 summarizes the results of respiration studies in the presence of CoA, DPN and TPN. It will be seen that CoA and DPN, given separately, stimulated respiration and that a mixture of both could substitute for yeast concentrate. As was to be expected, TPN could not fully replace DPN. Presence of niacinamide (10 mM), *co-carboxylase* (TPP, 1 mM), *flavin mononucleotide* (FMN, 1 mM), *flavin-adenine dinucleotide* (FAD, 1 mM), in the basic system containing ATP, cytochrome c and yeast concentrate (table 1), did not raise the O_2 consumption above the values obtained when the unsupplemented system was used. The same was true when the basic system was supplemented with *cystidine diphosphate* (CDP 1 mM), *guanosine diphosphate* (GDP 1 mM), or *inosine diphosphate* (IPP 1 mM). Glutathione (1 mM) did not raise net respiration but doubled endogenous respiration. *d, l-Thioctic acid* (0.017 and 0.033 mM), in the absence or presence of niacinamide and TPP, did not affect the rate of O_2 uptake. When tested for reasons not to be discussed, serotonin and heparin did not influence O_2 consumption of epithelial homogenate. Of inorganic factors the need for $MgCl_2$, $MnCl_2$ and $CaCl_2$ was investigated in a few experiments. A 2 to 4 mM solution of $MgCl_2$

TABLE 5

Effect of CoA, DPN and TPN on respiration of epithelial homogenate

Composition of reaction mixture is given in table 1. Final Versene concentration in Warburg flask 0.33 mM. Substrates: 4 mM fumarate+4 mM pyruvate. Temp. 35°C. All factors were given in 0.1 mM concentrations. Yeast concentrate (YC): 0.1%. All observations were made in pairs.

Substrate	YC	CoA	DPN	TPN	O_2 consumption	
					Total	Net
					µl/hr./200 mg wet tissue	
−	+	−	−	−	61	—
+	+	−	−	−	132	71
+	+	+	−	−	132	71
+	+	−	+	−	134	73
+	+	+	+	−	135	74
−	−	−	−	−	60	—
+	−	−	−	−	86	26
+	−	+	−	−	104	44
+	−	−	+	−	107	47
+	−	+	+	−	134	74
−	−	+	+	+	61	—
+	−	+	+	−	146	85
+	−	+	−	+	120	59
+	−	+	+	+	131	70

TABLE 6

Effect of substrate concentration on O_2 consumption of epithelial homogenate

Reaction mixture, see table 1. Final Versene concentration: 0.33 mM. Temp. 35°C. Average values based on two to 6 experiments for each combination of substrates.

Fumarate	Pyruvate	O_2 consumption	
		Total	Net
µMoles/3 ml	*µMoles/3 ml*	*µl/hr./200 mg wet tissue*	
0	0	62	—
3	0	126	64
6	0	153	91
12	0	160	98
12	3	180	118
12	6	195	133
12	12	203	141
12	18	189	127
12	24	195	133
18	0	164	102
24	0	171	109
48	0	160	98
96	0	139	77

d to optimal O_2 uptake when using fumate + pyruvate as substrates. $MnCl_2$ in nall concentrations (up to 0.16 mM) either increased nor decreased the rate f respiration. One to 2 mM solutions of nCl_2, however, inhibited O_2 uptake, as d 0.5 to 4 mM solutions of $CaCl_2$.

3. *Substrate concentration.* In the preeding experiments mixtures of 4 mM marate and 4 mM pyruvate were arbiarily employed. Table 6 summarizes the sults of experiments which were aimed at finding the optimal concentration for those substrates. It can be seen that best results were obtained when both fumarate and pyruvate were used in 4 mM concentrations. Since the 10% epithelial homogenate used in these experiments contained abundantly, though not exclusively particulate matter (see under methods), and because there was some latitude in the optimal concentrations for the two substrates, it is believed that results similar to those shown in table 6 may have

TABLE 7

Effect of various metabolites on O_2-consumption of epithelial homogenate

Reaction medium: see table 1. Temp. 35°C. Average values based on two to 8 experiments for each substrate or substrate combination.

	12 μMoles of each substrate in 3 ml	O_2 consumption	
		Total	Net
		μl/hr./200 mg wet tissue	
a	No substrate	40	—
b	Pyruvate	48	8
	Fumarate	161	121
	Fumarate + pyruvate	223	183
c	Succinate	162	122
	Succinate + pyruvate	227	187
d	Citrate	173	133
	Citrate + pyruvate	171	131
	Citrate + pyruvate + fumarate	199	159
e	α-Ketoglutarate	203	163
	l-Glutamate	221	181
	α-Ketoglutarate + l-glutamate	233	193

been obtained if the epithelium had been in a state of complete homogenization.

4. *Comparison of metabolites.* Several Krebs cycle metabolites, alone and in mixtures, and also l-glutamate were used as substrates. All of those tested led to increased oxygen consumption (table 7). The different qualities of the homogenates used in the different series of experiments (a through e) makes it impossible to compare quantitatively the effects of the various substrates on respiration. On the other hand, the data within each series are comparable because they were obtained with the same homogenate preparation. Pyruvate alone had little effect on rate of respiration. Fumarate, succinate and citrate greatly stimulated O_2 uptake. Further increases in O_2 uptake were seen when fumarate or succinate were used together with pyruvate. This was not the case with citrate + pyruvate. α-Ketoglutarate and *l*-glutamate proved to be two of the best substrates for skin epithelial homogenate. Phosphoglycerate and fumarate together were as effective as the mixture of pyruvate and fumarate. Phosphoglycerate, when given alone, was no more effective than pyruvate alone.

5. *Effects of inhibitors.* Sodium arsenite (0.02 mM) and fluoroacetate (4 mM) inhibited fumarate + pyruvate (4 mM solutions) oxidation by about 60% and 15% respectively. The rate of oxidation of *l*-glutamate or of α-ketoglutara or of a mixture of the two substrates (mM solutions) was reduced to about 70 of the control values when the solutio were poisoned with 4 mM malonate. T experiments were carried out with reactic mixtures of the composition mentioned table 1.

COMMENTS

The extensive work that has been do during the past three decades on acti transport of electrolytes and water acro isolated frog skin has contributed on little to an understanding of the metabol reactions which energize the transport. is generally assumed that active ion tra port in frog skin is linked with oxidati reactions. Leaf and Renshaw ('57b) ha found, however, that active Na^+ transpo continues under completely anaerobic co ditions though at a slower rate (20 40%) than in the presence of oxygen. is perhaps, reasonable to assume that bo the glycolytic pathway and the Krebs cyc are in operation in the epithelium of fr skin. Proof for this, however, is insuf cient. The results presented in this pub cation may be of interest for the followi reasons.

1. A simple method is described f complete separation of the epithelial lay from the corium in frog skin. No studi on cellular metabolism, meaningful in r lation to active electrolyte transport, cou

e made on whole, intact skin. For bio-
iemical investigations, scrapings of the
ithelial layers are probably adequate.
ttention may be called, however, to the
ossibility of obtaining sufficiently large,
ntact sheets of epithelium for transport
udies.

2. The presented metabolic studies on
olated epithelium and on epithelial homo-
enate have led to results which strongly
oint to the operation of the Krebs cycle
n this tissue. This conclusion derives
rom respiration studies with Krebs cycle
netabolites singly or in combinations
tables 3, 5 to 7) and from the effects of
oisons, administered with and without
ubstrates, acting on enzymes of the Krebs
ycle (fig. 2; paragraph 5, section B). The
bservation described above (table 7) that
itrate, α-ketoglutarate, succinate, and
umarate led to stimulation of O_2 consump-
ion of epithelial homogenate may indicate
hat through the operation of the Krebs
ycle, malate and oxaloacetate were
ormed for pyruvate formation, followed
y pyruvate consumption via the citric-
cid cycle. If this interpretation is valid,
t is not difficult to understand why pyru-
ate, as the only substrate present, did
ot or not greatly stimulate O_2 consump-
ion via the Krebs cycle. These and other
peculations will have to be tested by esti-
nating chemically or otherwise, interme-
liary metabolites and their rates of turn-
ver in epithelial homogenates.

Although our interest was primarily in
netabolism of frog skin epithelium, about
vhich little is known, several reports in
he literature were found dealing with
ntermediary metabolism of epithelium of
nammalian skin (rat, guinea pig, hu-
nan). Proof of the operation of the citric-
cid cycle in mammalian skin, it seems,
s now conclusive (Barron et al., '48; Her-
ey and Mendle, '54; Griesemer and Gould,
54; Gardenghi and Marsilli, '56; Cruik-
hank, Trotter and Cooper, '57; Cruik-
hank, Hersey and Lewis, '58).

3. A good homogenate of frog skin epi-
helium, comparable in quality to kidney
nd liver homogenate, is rather difficult
o obtain. The experiences gained and
lescribed in this paper may be useful in
uture attempts to improve the morpho-
ogical characteristics of epithelial homog-
enate. Such improvements will have to be made before meaningful fractionation studies can be undertaken. Work in this direction is now in progress for studies on oxidative phosphorylation in frog skin epithelial homogenate.

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An X-Ray Induced Block Preventing Cell Degeneration and Accompanying an Inhibition of Cell Division in Yeast

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Continuous low doses of x-radiation interrupt cell division in yeast cells, while growth continues. Presumably the radiation selectively blocks some necessary reaction and keeps it blocked, so that division cannot occur, at least during short experimental periods (Spoerl et al., '54; Spoerl and Looney, '58). Ionizing radiation may affect cellular reactions indiscriminately because of the non-specific way in which it is absorbed. However, with doses sufficiently small to reduce the number of detectable responses to a few only which result from a high sensitivity to radiation or radiation products, it is reasonable to hope that the sites affected might be uncovered. The dose used in the present studies was low in terms of any observable cell response, and recovery was high. Yeast cells have been reported to grow over long periods of time at equivalent or even higher doses (Welch, '57). Further, the consistency of the interference with division and the resulting formation of paired-cells (Spoerl et al., '54), along with a synchronized recovery of the capacity to divide (Spoerl and Looney, '59) argue for a highly sensitive and also specific site of injury. During attempts to uncover reactions involved in this inhibition of division, a markedly increased uptake of P^{32} by irradiated as compared to unirradiated cells was noted after the cells had been starved overnight (Spoerl et al., '59). Further studies have shown that this interference with division, or perhaps other injuries coincident with the division injury, results upon starvation in differences between irradiated and unirradiated cells in weight, permeability, viability, fermentation, and respiration, as well as P^{32} uptake. The data described below, support the concept of specific injuries and contribute to the attempt to uncover key points of injury.

MATERIALS AND METHODS

A previously used culture of *Saccharomyces cerevisiae* was handled in general as described earlier (Spoerl and Looney, '58; Spoerl et al., '59). Cells were grown overnight and irradiated the following day in growth medium during the exponential phase of growth. The two-hour period of irradiation (used in all experiments) was started when cultures were at a concentration of approximately 8×10^6 cells/ml. A Maxitron 250 kvp x-ray machine with 0.5 mm Cu and 1 mm Al added filtration was used to provide a radiation dose of 2.0 kr/hr as measured with a benzoate dosimeter. Following irradiation, cells were removed from the growth medium and washed twice with distilled water by centrifugation and then resuspended in buffer for immediate measurements, extracted for analyses, or resuspended in distilled water (0.5 of original culture volume, usually 50 ml) and incubated on a reciprocal shaker overnight (21 hours, average) at 28.5°C as the means of starvation. Starved cells were removed from the water suspension and washed with water once by centrifugation before resuspension in buffer or extraction. Unirradiated cells were handled in parallel except for the irradiation. Aliquots of suspensions taken for dry weight were dried overnight at 105°C. Viability (colony forming ability) was measured by diluting cells in sterile

distilled water, plating in triplicate on Wort agar and incubating at 30°C for 48 or 72 hours. Cells were counted in a standard hemocytometer. Buds approximately greater in diameter than one half the diameter of the mother cell were counted as cells.

Oxygen use and CO_2 production were measured at 30°C by standard direct manometric procedures (Umbreit et al., '49) with air or N_2 as the gas phase. The phosphate buffer used was M/15 KH_2PO_4(pH 4.5), and the TST buffer consisted of an equimolar mixture of tartaric and succinic acids adjusted to the desired pH with triethylamine (Rothstein et al., '59). When K^+ was added, KCl at M/15 concentration was included in the TST buffer. After washing, cells were resuspended (0.3 to 14.0 mg dry wt/ml, depending upon the measurement to be made) in buffer containing 0.1 M glucose and then transferred (2.0 ml) to the Warburg flasks. The pH was checked also after gas exchange measurements were completed. Time of handling was standardized (100 minutes average time to 0 reading after irradiation stopped). Oxygen use under endogenous conditions decreased after 100 minutes of readings; with glucose, rates increased somewhat during a 300 minute period of readings; anaerobically the CO_2 production rate was constant over a 200-minute period of readings.

Cells for analyses were chilled at the termination of the irradiation or after starving and subsequently handled and extracted cold (4°C or less). Fractionations of phosphorus (P) containing compounds and analyses for the radioactive P (P^{32}) uptake experiments were carried out as was done previously (Spoerl et al., '59). The P data presented in table 1 were obtained by a modified procedure, also similar to one used previously (Spoerl and Looney, '58) except for additional measurements with the trichloroacetic acid (TCA) extract. In the present experiments, the cold TCA extract was immediately analyzed colorimetrically for ortho P and again analyzed for ortho P after hydrolysis at 100°C for 10 minutes in 1 N HCl. The ortho P resulting from hydrolysis is listed as labile P (e.g., soluble polyphosphates (PP), trimetaphosphate, pyrophosphate, adenosine triphosphate). Th difference in P content between total TC extract P and the ortho P determined afte HCl hydrolysis is listed as stable P (e.g adenylic acid). Nitrogen was measure by digestion and nesslerization as was don previously (Spoerl and Carleton, '54), an carbohydrate, by the anthrone method a used previously (Spoerl and Looney, '58a

P^{32} uptake was measured in cells whic had been suspended in succinate buffe (50 ml, 0.05 M) containing glucose (0. M) and incubated for 30 minutes. P^{32} i H_3PO_4 was then added to make a fin 0.02 M P concentration (final pH, 4.9 and samples taken for analysis after add tional periods of incubation. The P^{32} co tent of the various cell fractions was d termined in the solutions resulting fro the colorimetric P analysis by counting i an annulus-type liquid counting tube (R diation Counter Laboratories, model 71

RESULTS

P^{32} uptake. The increased uptake P^{32} by starved irradiated as compared to u irradiated cells is shown in figure 1. Th markedly greater overall uptake by irrad ated cells did not occur when uptake w measured immediately after irradiatio (Spoerl et al., '59). Additional analys indicated increased amounts of total P p unit of dry weight in the irradiated cell during the period of P^{32} uptake measur ments, but very little if any increase in u irradiated cells. This observation was n unequivocal, since P uptake could ha been concealed by a greater increase in d weight of unirradiated cells. Amounts insoluble PP increased in both types cells, and ortho P decreased. The total content of irradiated cells also appear to be greater than that of unirradiated cell immediately after starving and before was added for uptake measurement.

P composition. To substantiate som of the above apparent differences in P co tent, and with the hope that other diffe ences might be found, perhaps related observed differences in P loss (see below which would provide an insight into th cause for the greater P^{32} uptake, a series analyses was made of irradiated and u irradiated cells immediately after irradi tion and again after a period of starvatio

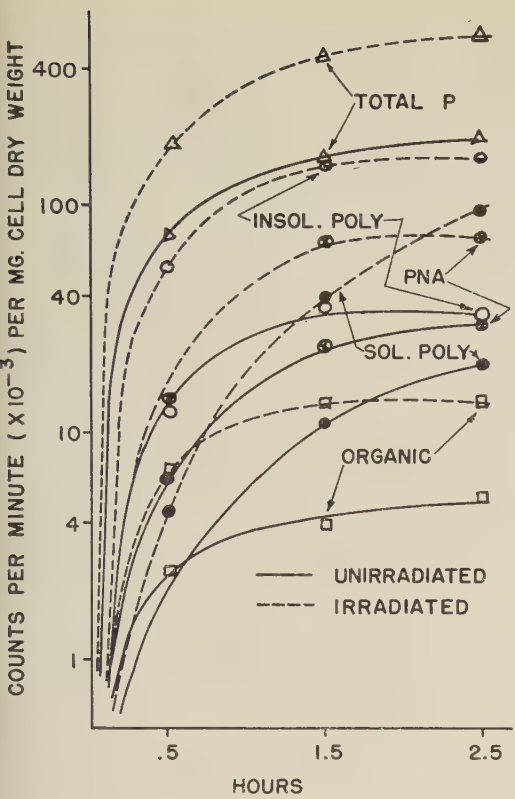

Fig. 1 P^{32} content of unirradiated and irradiated yeast cells which were starved and then suspended in buffer to which P^{32} was added. Total P^{32} and the P^{32} content of several phosphorus containing cell fractions are shown at different times after the addition.

The data (listed in table 1) agree with previous measurements in showing a significant increase in insoluble PP immediately after irradiation (Spoerl and Looney, '58). Other P constituents showed no significant changes in these cells. It is conceivable in the case of soluble PP (labile P fraction) that no decrease in these compounds was observed in the present experiments because molecules of all sizes were measured by the analytical procedure involving hydrolysis, whereas in other studies where a decrease in amount was observed (Spoerl et al., '59) smaller molecules or fragments somehow resulting from irradiation were not precipitated and measured by the method of analysis used. Alternately, other labile P compounds may have increased in amount to compensate for a PP decrease.

Starved unirradiated cells, on the other hand, showed a number of changes. Nucleic acids were increased in amount per unit of dry weight, significantly for pentosenucleic acid (PNA). The increase was correlated with a decrease in dry weight (see table 2) and evidently is explained by this weight decrease if it is assumed that no breakdown of formed nucleic acid occurred. Insoluble PP were reduced to the vanishing point by breakdown to ortho P as has been observed in starved cells by Wiame ('49) and others. Labile P, normally largely soluble PP, maintained its

TABLE 1

Phosphorus content of unirradiated and irradiated yeast cells, immediately after the period of irradiation and after an additional period of starvation

P fraction	Immediate unirradiated	Per cent of immediate unirradiated[2]		
		Immediate irradiated	Starved unirradiated	Starved irradiated
	mg/100 mg dry weight			
Total	2.80 ±0.022	102 ± 0.79	98.8± 4.58	107 ± 1.25[1]
PNA	1.25 ±0.015	98.7± 1.31	112 ± 2.94[1]	105 ± 2.22
DNA	0.28 ±0.002	111 ±14.6	130 ± 8.90	94.8± 9.68
Insol. PP	0.280±0.025	143 ±10.4[1]	4.1± 2.62[1]	12.0± 5.45[1]
Phospholipid	0.169±0.004	98.7± 2.44	78.7± 4.24[1]	87.6± 1.27[1]
Total TCA extract	0.961±0.010	97.0± 3.03	125 ±11.1	143 ± 7.03[1]
Ortho	0.338±0.008	95.7± 2.39	253 ±24.2[1]	283 ±15.8[1]
Labile	0.455±0.010	96.8± 5.94	94.9±14.6	98.6±11.4
Stable	0.168±0.010	99.3± 3.34	44.3± 1.32[1]	61.4± 4.94[1]

[1] Differs significantly from 100 at the 5% level.

[2] Means plus or minus standard errors are calculated from three to 7 separate experiments each analyzed in duplicate. Most of the means for immediate cells are based on 7 separate experiments; most of the means for starved cells are based on 4 separate experiments.

TABLE 2

Changes in unirradiated (Unir.) and irradiated (Ir.) yeast cell constituents during starvation

Dry weight loss (%)	Loss to suspending water			
	P		N	
	% of cell dry weight	% of cell P content	% of cell dry weight	% of cell N content[2]
Unir. 15.5 ± 1.2(54)[1]	0.307 ± 0.036(11)	11.0	0.92 ± 0.09(4)	9.2
Ir. 13.8 ± 1.0(48)	0.124 ± 0.008(8)	4.4	0.60 ± 0.03(6)	6.0
Ir. ÷ Unir. 0.85	0.40	0.40	0.65	0.65

[1] Means plus or minus standard error. Figure in parentheses lists number of separate experiments each involving duplicate samples.

[2] Cell N content taken as 10%.

prestarved level, while the stable organic P fraction decreased.

Starved irradiated cells showed some additional differences in response. Total P per unit dry weight was significantly increased and the P content of the TCA extract was greater probably due to a larger stable P fraction. The increased total P content evidently was the result of a dry weight loss rather than additional uptake from cells which might have lysed during starvation since the number of cells in the suspension did not decrease significantly (table 5). Unirradiated cells did not show a similar increase in total P because of a greater loss of P from these cells (table 2). Nucleic acids did not increase as in unirradiated cells probably because of a lesser loss in dry weight (table 2). Phospholipids decreased in both types of starved cells presumably through utilization as an energy source. Thus, irradiated cells showed what appeared to be a lesser amount of breakdown of P compounds and a smaller P loss than did unirradiated cells.

Constituent changes. Measurements made with the P^{32} uptake experiments showed a greater loss of P to the suspending water from unirradiated than from irradiated cells during the period of starvation. This observation, in addition to raising the question of what related P changes (above) might occur, also suggested that other measurements be made to uncover possible losses in dry weight, carbohydrate, or nitrogen (N). The losses of P and N (table 2) by unirradiated cells appeared to be unusual for yeast but evidently can be accounted for by culture age (see below). Carbohydrate analyses showed no significant differences due to starvation or to irradiation (Immediate: Ir./Unir. = 96.9 ± 2.2(8); Starved: Ir./Unir. = 96.9 ± 2.1 (5)).

Manometric studies. Measurements of oxygen use (summarized in table 3) in succinate buffer identical to that in which P^{32} uptake was measured showed an increased oxygen use by starved cells. Succeeding measurements were made in phosphate or TST buffer. Starved irradiated cells took up more oxygen than unirradiated cells both in phosphate buffer and in TST buffer at pH 4.5. Cells in TST buffer (pH, 4.5) used less oxygen than in phosphate buffer. Further, uptake in TST buffer when measured immediately was significantly less for irradiated cells. These decreased usages were repaired and increased even above the level of use observed in phosphate buffer by the addition of K^+.

In addition to differences in oxygen use due to the buffer employed, an altered response occurred also at a higher pH. Thus, oxygen use in TST buffer at pH 5.0 was significantly reduced in irradiated cells when measurements were made immediately and was similar to rather than greater than that of unirradiated cells following starvation. A similar response, i.e., a reduced production by irradiated cells immediately following irradiation and a similar production after starvation, was found also for carbon dioxide formation in TST buffer at pH 5.0. Evidently leakage or other cell injury resulting from irradiation was affected by pH in this buffer. Cells were not preconditioned by pH during starvation, since both unirradiated and irradiated cell suspensions showed the same pH (7.5) following starvation.

Irradiated cells used less oxygen than unirradiated cells during endogenous me-

tabolism. Endogenous R.Q. values of approximately 1.0 were obtained for both types of cells. R.Q. values for cells in phosphate buffer (pH, 4.5) with glucose immediately after irradiation were alike (4.4) and were reduced upon starvation though relatively less for irradiated cells (3.8 Ir.; 3.0 Unir.). In TST buffer at pH 5.0, the R.Q. of cells immediately after irradiation was 3.7 and of unirradiated cells, 2.5, whereas after starving both types of cells showed values of 1.9.

In general starved cells used oxygen at a lower rate (μl/mg/hr) than before starving. A marked difference was evident with unirradiated cells (table 3). Bair and Stannard ('55) with cells from mature yeast cultures had observed Q_{O_2} values after starvation similar to those before. Consequently, additional observations were made with cells from mature cultures grown to a maximum cell count ($1–2 \times 10^8$/ml) in contrast to the usual exponential phase cultures. Obviously, though an equivalent dose of radiation could be given, division could not be interrupted, since these cells were no longer dividing. A two-hour 2.0 kr/hr irradiation produced cells (table 3, phosphate buffer "(old)") which showed no effect on oxygen use due to irradiation and which used oxygen after starvation at a rate similar to that of cells before starvation. This lack of response in "old" cells to irradiation or starvation occurred also in the case of carbon dioxide production (table 4). Also, the percent of dry weight loss upon starvation did not differ between irradiated and unirradiated cells (8.20 ± 1.06 Ir.; 8.00 ± 0.84 Unir.; 4 experiments), differences in viability of the two types of cells were not evident in the few measurements made (Ir. ÷ Unir. 106 ± 3.6; 3 experiments) and P and N losses were reduced and similar (2 experiments).

Anaerobic carbon dioxide production (see table 4), like oxygen use, was greater in starved irradiated than in unirradiated cells, and was generally less in starved cells, markedly so in some cases. When production by irradiated cells was measured immediately in TST buffer (pH, 4.5), a lower rate was observed than for unirradiated cells. Again, this rate was increased by the addition of K^+ to a rate greater than that observed in phosphate

TABLE 3

Oxygen use (Q_{O_2} values) by unirradiated (Unir.) yeast cells (handled in parallel with irradiated cells) and x-irradiated (Ir.) yeast cells immediately after irradiation and after a period of starvation in distilled water

Buffer	Immediate			Starved		
	Unir.	Ir.	Ir. ÷ Unir. × 100	Unir.	Ir.	Ir. ÷ Unir. × 100
Succinate (4.9)[1]	79.3 (1)	86.9 (1)	110 (1)	56.1 ± 4.1 (3)[2]	88.4 ± 20 (3)	154 ± 27 (3)
Phosphate (4.5)	79.5 ± 4.0 (14)	84.9 ± 4.4 (14)	108 ± 5.4 (14)	41.7 ± 5.3 (14)	57.9 ± 4.6 (14)	152 ± 11 (14)[3]
Phosphate (4.5) (endogenous)	5.50 ± 0.24 (4)	4.87 ± 0.39 (4)	88.2 ± 3.6 (4)[3]	ca 1.8	ca 1.2	
Phosphate (4.5) (old)	87.7 ± 3.8 (3)	83.1 ± 3.0 (3)	94.8 ± 1.3 (3)	81.6 ± 0.8 (3)	80.7 ± 1.7 (3)	98.8 ± 1.5 (3)
TST (4.5) (old)	71.0 (2)			69.6 (2)		
TST (4.5)	48.7 ± 5.0 (4)	36.5 ± 6.1 (4)	73.8 ± 4.2 (4)[3]	27.3 ± 1.9 (3)	38.6 ± 4.7 (3)	140 ± 8.0 (3)[3]
TST + K^+ (4.5)	93.6 ± 3.6 (4)	94.4 ± 3.5 (4)	100 ± 1.5 (4)	43.1 ± 1.4 (4)	68.4 ± 5.3 (4)	159 ± 12 (4)[3]
TST + PO_4 (4.5)	43.2 (2)	48.6 (2)	99.1 (2)	10.1 (2)	22.2 (2)	291 (2)
TST (5.0)	45.6 ± 2.4 (3)	17.7 ± 2.5 (3)	39.6 ± 7.5 (3)[3]	31.5 ± 5.3 (4)	27.7 ± 1.4 (4)	95.6 ± 16 (4)

[1] Figure in parentheses in buffer column is pH.

[2] Means plus or minus standard error. Figure in parentheses lists number of separate experiments each containing duplicate flasks. Q_{O_2} values with glucose are for the period of 100 to 200 minutes of readings; endogenous values are for the period 0 to 100 minutes. Zero time averaged 100 minutes after irradiation was stopped.

[3] Differs significantly from 100 to 5% level.

TABLE 4

Carbon dioxide production by unirradiated (Unir.) yeast cells (handled in parallel with irradiated cells) and x-irradiated (Ir.) yeast cells immediately after irradiation and after a period of starvation in distilled water

Buffer	Immediate			Starved		
	Unir.	Ir.	Ir. ÷ Unir. × 100	Unir.	Ir.	Ir. ÷ Unir. × 100
			Anaerobic ($Q_{CO_2}^{N_2}$)			
Phosphate (4.5)[1]	329 ± 16(7)[2]	327 ± 15 (7)	99.7 ± 3.0(7)	123 ± 17 (7)	236 ± 14 (7)	206 ± 19 (7)[3]
Phosphate (4.5) (old)	303 ± 14(3)	292 ± 7.0(3)	96.9 ± 4.2(3)	315 ± 8.2(3)	314 ± 5.9(3)	99.7 ± 0.63(3)
TST (4.5)	193 ± 16(3)	148 ± 18 (3)	75.8 ± 5.2(3)[3]	32.2 ± 7.4(4)	78.2 ± 25 (4)	235 ± 23 (4)[3]
TST + K^+ (4.5)	435 ± 28(3)	413 ± 13 (3)	95.2 ± 2.9(3)	140 ± 24 (3)	252 ± 25 (3)	187 ± 20 (3)[3]
TST + PO_4 (4.5)	143 ± 36(3)	57.2 ± 3.9(3)	44.4 ± 10 (3)[3]	20.8 ± 8.6(3)	12.8 ± 5.3(3)	64.4 ± 7.6(3)[3]
TST (5.0)	134 ± 23(4)	112 ± 19 (4)	84.8 ± 4.6(4)[3]	77.7 ± 8.2(3)	67.9 ± 14 (3)	87.9 ± 4.1(3)
			Aerobic Fermentation ($Q_{CO_2}^{air} - Q_{O_2}$)			
Phosphate (4.5)			81.9 ± 14 (11)		138 ± 14 (11)	214 ± 28 (11)[3]

[1] Figure in parentheses in buffer column is pH.

[2] Means plus or minus standard error. Figure in parentheses lists number of separate experiments each containing duplicate flasks. Rate values were constant over a 200 minute period of measurement. Zero time averaged 100 minutes after irradiation was stopped.

[3] Differs significantly from 100 at 5% level.

buffer. Phosphate ion added to the TST buffer did not repair the decreased carbon dioxide production, but resulted in a further decrease, relatively greater in irradiated cells both immediate and starved. This phosphate ion effect was consistent with the apparently greater oxygen use and carbon dioxide production observed for cells in TST buffer plus K^+ compared to cells in phosphate buffer. Aerobic fermentation ($Q_{CO_2}^{air} - Q_{O_2}$) values also showed a higher rate by starved irradiated cells than by unirradiated cells.

In an attempt to evaluate differences in terminal pathways of respiration, gas exchange also was measured with sodium pyruvate (0.1 M) and sodium lactate (0.1 M) as substrates in place of glucose (phosphate buffer, pH, 4.5). A generally greater oxygen uptake with pyruvate occurred with starved irradiated cells, but uptake varied from 20 to 70 µl/hr/mg in different experiments with irradiated cells and from 7 to 30 for unirradiated cells. Lactate was used at a higher average rate (ca 65 µl/hr) and with only a slightly greater (5µ) usage by irradiated cells. These data do not provide for clear-cut conclusions because of the great variability encountered, especially with pyruvate, but apparently terminal systems also have a greater capacity in irradiated cells.

Viability. Constitutent losses and relatively decreased respiratory capacities of unirradiated starved cells suggested that more of these cells than irradiated cells may have died and lysed as a result of starvation. However, viability measurements did not indicate large differences. Cells plated immediately after irradiation were 12% less viable than unirradiated cells, whereas after starvation irradiated cells were 23% more viable than unirradiated cells. Buds on some unirradiated cells may have enlarged and increased the cell count slightly during starvation, and a few irradiated cells may have lysed during the same period, but changes in cell number were not significant (table 5). Measurements of paired cells indicated some splitting during starvation of unirradiated cells but little change in irradiated cells.

Exponential phase cells have many buds and irradiated cells are paired, consequently cell units (units consisted of singl

TABLE 5

Viability of cells, numbers of cells and numbers of paired cells in unirradiated (Unir.) and irradiated (Ir.) yeast cultures immediately after the period of irradiation (Im.) and after an additional period of starvation (St.)

Viability	Viability		Number of cells		Paired cells (% of total)	
					Immediate	Starved
	% of *unirradiated*	% of *immediate*		% of *immediate*		
Im.	88.5 ± 3.5(15)[1,3]	Unir. 71.3[2,3]	Unir.	105 ± 2.6(19)	Unir. 56.5 ± 1.52(21)	44.8 ± 1.41(17)
St.	123 ± 7.7(7)	Ir. 97.5	Ir.	97.5 ± 2.8(19)	Ir. 90.0 ± 0.45(21)	86.2 ± 0.62(17)

[1] Means plus or minus standard error. Figure in parentheses lists number of separate experiments.
[2] Calculated from means rather than individual values.
[3] Differs significantly from 100 to 5% level.

cells, paired cells or larger groups of cells each of which could produce only one colony on a plate) rather than total cells were counted in viability determinations. If one cell of a pair was viable and one not, viability data based on cell units would be biased. To evaluate such an occurrence cells were plated on grid marked agar, counted at succeeding times and the counts recorded by group size. If only one cell of a pair is viable a three-cell group will result from the first division, whereas if both are viable a four-cell group will result. The very small percentage of cells present at any time in groups of three (table 6) shows that in almost all cases both cells of a pair divided (generation time of these cells normally is approximately 1.5 hours). Cells not in multiple groups at 8.5 hours indicated that 7% were non-viable. Because the percentage of non-viable cells was low, these data are not as critical as they would be if based upon cells showing a greater reduction in viability. In addition, possible deaths after a number of divisions had occurred (cells in multiple groups) were not estimated, and conceivably some division delays which extended up to 8.5 hours may still have been overcome at a later time. Within these limits or deficiencies of measurement it may be concluded that the viability figure given above (table 5) is valid and is not greatly less than it should be.

DISCUSSION

The above observations describe a greater breakdown of unirradiated, control cells than of irradiated cells during a period of starvation. Irradiation preceding starvation evidently blocked some reaction or reactions which normally allow cell breakdown as shown by changes in permeability, viability, P^{32} uptake and respiratory capacity. In addition, an evidently decreased K^+ retentivity and a small reduction in endogenous respiration was observed immediately after irradiation. An effect upon K^+ permeability has been reported previously by Bair and Stannard ('55) who also found an effect of starvation opposite the one described here, an enhancement of radiation injury when cells were starved before irradiation.

TABLE 6

Cell division and growth of yeast cells plated on Wort agar immediately after irradiation

Hour[1]	Number of cells per colony (per cent)					Total cells
	1	2	3	4	multiple	
0.5	1.2	83.9	0.9	12.2	1.8	797(5)[2]
2.5	0.5	41.0	0.8	38.4	19.3	742(5)
4.5	0	14.9	0.4	24.8	59.9	706(5)
6.5	0	4.3	0.5	11.0	84.2	372(3)
8.5	0	2.0	0.4	4.3	93.3	255(2)

[1] Time after plating.

[2] Figure in parentheses lists number of separate measurements totaled to obtain data shown.

The prevention of breakdown by irradiation occurred in young exponential phase cells and not in old mature phase cells. This was demonstrated for oxygen use, carbon dioxide production, dry weight loss and viability, and presumably may hold also for other changes. A reduced injury by radiation of processes of growth and cell division was noted previously in cells of increasing age (Spoerl et al., '54). Differences between young and old cells are commonly recognized and are frequently reported. Thus with yeast, Eaton and Klein ('57) ascribed differences in acetate oxidation to an adaptation process, and an instability in young cells, expressed by wide variations in the rate of endogenous respiration, has been noted by Speigelman and Nozawa ('45). For other cells, Swann ('58) has reviewed rather thoroughly the varied differences in activity which may exist between dividing and mature cells. In the present case apparently the radiation affected some process, or processes, so that it no longer possessed the lability normally shown in young cells, nor the capacity to allow division to occur, but was stabilized to a condition similar to that in old cells which did not show the breakdown during starvation described above for young cells. This response is suggestive of possible parallelisms with the "aging" effect of radiation upon mammals.

The immediate effect of irradiation observed in the decreased rate of endogenous respiration indicates, because this respiration for most strains of yeast is entirely aerobic (Speigelman and Nozawa, '45) and because exogenous glucose oxidation was not reduced, an interference with an alternate oxidative route such as the hexosemonophosphate shunt or a decrease in amount or availability of substrate. Measurements of total carbohydrate and a chromatographic comparison of the trehalose content of a TCA extract indicated no differences between unirradiated and irradiated cells. However, these measurements probably were not adequately sensitive to allow a distinction to be made. If it is considered that an effect upon a shunt pathway occurred, this together with the slight indication of increased oxygen use with glucose and K^+ present (table 3) suggests that it would not be surprising to find some uncoupling of phosphorylation as has been observed with other cells by Maxwell and Ashwell ('53). Measurements showing the continued formation of polyphosphate (Spoerl et al., '59) need not contradict this possibility, since the degree to which the effect occurred evidently was not extensive.

Stimulation of oxygen use and carbon dioxide production by added K^+ was observed in both irradiated and unirradiated cells, but before starving it was greater for the irradiated cells (Relative rates: Unir. O_2 use 1.9, CO_2 production 2.2; Ir. O_2 use 2.6, CO_2 production 2.8 (tables 3 and 4)). Repair of the radiation effect which allows leakage of K^+ has been described previously (Bair and Stannard, '55) and Bruce ('58) has measured K^+ loss from irradiated cells. In these cases the radiation dose was much larger than that used in the present experiments. Because K^+ loss occurred in the present experiments with a drop of only 12% in viability, the 5:1 dose ratio for retentivity to viability effects, observed by Bruce ('58), apparently does not hold for these young growing cells. K^+ also stimulated starved cells, the effect being about the same for oxygen uptake by both unirradiated (1.6) and irradiated

(1.8) cells, whereas carbon dioxide production was increased to a greater extent in unirradiated (4.3) than in irradiated cells (3.2). Evidently during starvation new K^+ or other equilibria were established so that irradiated cells no longer showed the deficiency when compared to unirradiated cells which was observed before starvation.

Another change in permeability, resulting in this case from starvation, was observed in the greater loss of cell contents from unirradiated cells. The amounts of P and N lost from the cells were no more than the amounts normally extractable by TCA (Spoerl and Looney, '58) and consequently could have been primarily soluble intermediates. However, within certain limits at least, ortho P did not leak more readily from the cells. Rather it was markedly increased during starvation as part of an increase in total TCA extractable P material (table 1). Changes in the number of cells during starvation (table 5) did not indicate a lysis which could account for the greater amounts of P and N found in the water in which unirradiated cells were starved.

The striking consequences of irradiation observed following starvation (increased O_2 uptake and CO_2 production, loss of cell contents and reduced viability) were independent of K^+ loss, since they occurred both in the presence or absence of K^+. Whether or not they resulted from one radiation block is not easily evident and probably is more unlikely than likely. It is evident that some at least are interrelated such as the preservation of carbon dioxide producing capacity and the capacity to take up P^{32} which processes Goodman and Rothstein ('57) have shown to be related. Certainly the possibility exists that one block only was responsible for the observed changes.

The decrease in viability of unirradiated cells during starvation probably was a secondary effect. In cells unaffected by an injurious agent, e.g., unirradiated cells here, a reduced viability would result from, rather than precede some other deterioration. The relative decrease in viability was only 23%, whereas carbon dioxide production, relatively, was 50% less after starvation. Thus, the preservation of viability in starved irradiated cells would appear to be related to the protection of some other process. On the other hand, the action of injurious agents such as radiation may concurrently affect viability and other processes to different degrees as Gonzales and Barron ('56) have emphasized. This was observed here in unstarved irradiated cells by no reduction in rates of respiration or fermentation, or P^{32} uptake (Spoerl et al., '59), though viability was reduced.

A membrane change during starvation, somehow blocked by irradiation might possibly link the various effects described including the loss of cell contents. Such a change might be either a reduced retentivity only or also involve carrier systems participating in transport mechanisms. A membrane deficiency slowing glucose uptake could be reflected in oxygen use, carbon dioxide production and P^{32} uptake. The leakage of soluble metabolites from the cell would account for P, N and weight losses, as well as reduced viability at an advanced stage of loss. Additionally, reduced oxygen use, P^{32} uptake and carbon dioxide production might result directly from such a loss or as the result of an associated effect upon glucose transport. The partial loss of these capacities by irradiated cells during starvation (complete preservation was not observed in young cells for any process, except perhaps viability) would be related to the partial loss in these cells also of cell constituents (table 2).

Alternately, complete leakage of acid soluble materials from some, perhaps the non-viable cells, but not from all cells might account for the P and N loss. The dry weight loss observed probably would require that more than the acid soluble material was lost from 25 to 30% (i.e., non-viable) of the cells, and both the P and N losses approach the maximum possible from this portion of cells. Another factor to be considered is the possibility of breakdown and loss of additional N components of non-viable cells. If normally soluble compounds only were leaked from the cells these might have been replaced by synthesis after the cells were plated on Wort agar rather than resulting in non-viability. The loss of material which also occurred from irradiated cells, instead of indicating

a normal loss from any starved cell, might similarly be accounted for as complete leakage during starvation from those cells (12%) found non-viable immediately after irradiation.

The relatively high degree to which the capacity to produce carbon dioxide was preserved by irradiation; and conversely the marked reduction of this capacity in unirradiated cells, suggests the possibility of a selectively related action by radiation. Rothstein, et al. ('59) have examined and discussed the structural location of the fermentation apparatus in yeast and conclude that it is located in a relatively small peripheral region. Though the mitochondria of yeast tend to be located peripherally (Agar and Douglas, '57), these structures normally contain terminal pathway enzymes and appear to be radiosensitive rather than resistant (Noyes and Smith, '59). Rothstein ('54) also has summarized evidence indicating that two separate glycolytic systems exist, the anaerobic being the large component. This system could be adjacent to the cell membrane, or be some part of it as has been suggested for respiratory systems of other cells (Mathews and Sistrom, '59). It could be speculated then, that some action of radiation altered the membrane by stabilizing it, perhaps primarily the glycolytic component or adjacent structure or perhaps also a mechanism involved in retentivity, against the deleterious changes which occur during starvation and thus produced the preservation of various capacities as observed.

Though the site of the induced radiation block or blocks has not yet been pinpointed and considerable speculation is needed to interpret the present results, the observation of a functional block in young cells revealed by starvation, the measurement of concomitant changes and the implications discussed advance the attempt to locate a key effect. The relation between the block which stabilized glycolysis to a condition more like that which occurs in "old" cells and an apparently concomitant interference with cell division, a condition also occurring in "old" cells, is of considerable interest for our understanding of the inhibition of cell division. Sites other than nucleic acid formation (Spoerl and Looney, '58) quite evidently are involved in an inhibition of division by radiation and certainly need elucidation.

SUMMARY

Growing yeast cells given a low dose of x-radiation adequate to inhibit cell division were found, after a period of starvation, to be more viable, more capable of taking up P^{32} and oxygen and of producing carbon dioxide than were unirradiated cells. The possibility was discussed, that the action of radiation was somehow to stabilize the cell membrane or a component structure and thus preserve these capacities through the period of starvation as well as prevent a loss of phosphorus and nitrogen compounds such as occurred from unirradiated cells. The starvation responses occurred in young cells but not in old cells. Additionally, a reduced potassium retentivity and rate of endogenous respiration were observed immediately after irradiation.

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